Remarks

Claims 45-69 were pending in the subject application. Claim 65 remains pending but withdrawn from consideration. Accordingly, claims 45-69 are currently before the Examiner for consideration. Favorable consideration of the pending claims is respectfully requested.

Applicants acknowledge that claim 65 has been withdrawn from further consideration as being drawn to a non-elected invention. Applicants reserve the right to rejoinder of any non-elected process claims upon an indication of an allowable product claim in accordance with MPEP §821.04.

Claims 45-64 and 66-69 are rejected under 35 USC §112, first paragraph, as lacking sufficient written description. The Examiner asserts that the specification does not provide an adequate written description of the polynucleotide encoding a Human Leukocyte Antigen (HLA) binding fragment of SEQ ID NO:1, comprising at least five consecutive amino acids of SEQ ID NO:1, or the polynucleotide that is complementary along the full length of the polynucleotide encoding the HLA binding fragment. Applicants respectfully submit that the specification conveys with reasonable clarity to those of ordinary skill in the art that, as of the application's date, Applicants were in possession of the claimed subject matter.

The Examiner cites immunology textbooks such as Cruse *et al.* (Illustrated Dict. Of Immunology, 2nd ed., CRC Press, 2003) for teaching that

> ...an epitope or antigenic determinant interacts with its corresponding antibody based on the three-dimensional structure of both molecules and the fit between them... These epitopes may be conformational (or discontinuous) epitopes which are formed from separate regions in the primary sequence that are brought together by proper folding...

This is correct in the context of epitopes for antibodies; however, the present claims are drawn to polynucleotides encoding HLA binding peptides, and these peptides are linear, as evidenced by pages 11-17 of Fikes J. *et al.* (see lines 1-3 at page 12 of Chapter 2 in Morse M.A. *et al.*, Handbook of Cancer Vaccines, Humana Press: Totowa, New Jersey, 2004) and pages 671-692 of Bradley J.A. *et al.* (see paragraph bridging pages 675-676 of Chapter 43 in Majid A.A. *et al.*, Advanced Surgical Practice, Greenwich Medical Media: London, 2003). Therefore, the influence of protein folding on

antibody-antigen interactions is not relevant to the binding requirements of HLA binding peptides.

The claimed HLA binding fragments constitute a well defined number of peptides which can be directly excised from SEQ ID NO: 1. Hence, the provision of the entire sequence of SEQ ID NO: 1 provides the necessary starting point for a very simple test where fragments are assayed for their binding to HLA molecules. Given the high level of skill in the polypeptide art and the disclosure of SEQ ID NO:1 in the specification, those of ordinary skill in the art would consider the Applicants to have been in possession of the entire breadth of the claimed genus of polypeptides at the time the application was filed. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

Claims 66-69 are also rejected under 35 USC §112, first paragraph, as new matter. Applicants respectfully submit that claims 66-69 are not new matter. At page 10 of the Office Action, the Examiner indicates that the phrase in question does not appear in the specification or claims as originally filed. Applicants pointed out support for claims 66-69 at page 6, third paragraph, of Applicants' Amendment submitted to the Patent Office on March 5, 2009. Specifically, support for claims 66-69 can be found, for example, in paragraph [0095] at page 41 of the specification. Accordingly, reconsideration and withdrawal of the rejection under 35 USC §112, first paragraph, is respectfully requested.

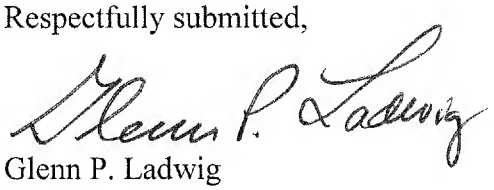
It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachment: Fikes J. *et al.* "Chapter 2: The Rational Design of T-Cell Epitopes With Enhanced Immunogenicity" In: Morse M.A. *et al.*, Handbook of Cancer Vaccines, Humana Press: Totowa, New Jersey, 2004, pp. 11-17.
Bradley J.A. *et al.* "Chapter 43: Transplantation Immunology" In: Majid A.A. *et al.*, Advanced Surgical Practice, Greenwich Medical Media: London, 2003, pp. 671-692.

2 The Rational Design of T-Cell Epitopes With Enhanced Immunogenicity

John Fikes, PhD

CONTENTS

1. THE CHALLENGE OF OVERCOMING IMMUNOLOGICAL TOLERANCE

For all cancer vaccine strategies, a major challenge facing efforts to induce a clinically effective T-cell response is the necessity to break tolerance to normal, "self" antigens. To control auto-reactivity, some T cells with high avidity for tumor-associated antigen (TAA) epitope–major histocompatibility (MHCs) complexes are deleted in the thymus and the remaining T cells are controlled by peripheral tolerance *(1)*. However, several groups have demonstrated using in vitro systems that thymic-deletion of TAA-specific cytolytic T cell (CTL) is not complete *(2–4)*. More importantly, it is clear that in some patients, natural exposure to tumor or immunization with wild-type antigens or epitopes can induce CTL of sufficient avidity and functionality to infiltrate tumors in vivo and/or recognize tumor cells in vitro. Therefore, although the fundamental vaccine strategy of targeting TAA to mount tumor-specific immune responses is supported, it remains a significant challenge to design cancer vaccine strategies that consistently overcome immunological tolerance in order to effectively activate and maintain therapeutic T-cell responses. Experimentation in the late 1980s and 1990s has resulted in a detailed understanding of the molecular mechanisms controlling T-cell activation and effector function. It is now appreciated that the interaction of a T-cell receptor with a peptide epitope presented by an antigen-presenting cell (APC) in the context of an MHC molecule generates the central event (referred to as "signal 1") in the activation of naïve or memory

From: *Handbook of Cancer Vaccines*
Edited by: M. A. Morse, T. M. Clay, and H. K. Lyerly © Humana Press Inc., Totowa, NJ

T cells. Studies have also demonstrated that the specificity and affinity of peptide binding by human lymphocyte antigens (HLAs) is determined by the interactions between the side chains of the linear peptide epitope and the residues present in each HLA molecule. Insights from these studies have given rise to two strategies for the rational modification of T-cell epitopes to enhance immunogenicity and "breaking" of immunologic tolerance. The molecular mechanisms, immunological consequences, and the cancer vaccine applications of each approach will be discussed.

2. FIXED-ANCHOR EPITOPE ANALOGS

One type of modification that can be utilized to enhance the immunogenicity of wild-type TAA-derived peptides involves the substitution of a single amino acid residue to facilitate an increase in the binding affinity of the analog peptide for an HLA molecule. Although the affinity of peptide binding by an HLA molecule is essentially the product of the interactions between residues of the peptide and the residues in the peptide-binding cleft of the HLA molecule, two positions have a dominant influence on binding, and are referred to as "primary anchor positions." For most HLA-binding peptides, these primary anchor residues are position 2 and the carboxyl-terminus. The specific amino acids that most effectively function as primary anchor residues in a peptide are dependent on the composition of the binding pocket of the given HLA molecule, and the amino acids that are "preferred" and "tolerated" at each peptide position have been defined for numerous HLA molecules *(5)*. For example, for binding to HLA-A2.1, L,M and V,I,L are preferred anchors at position 2 and the C-terminus, respectively. Knowledge of these HLA-specific patterns, referred as motifs, is the foundation for the rational design of fixed-anchor analog epitopes. The primary amino acid sequence of a wild-type TAA can be analyzed using motif-based algorithms to identify peptides that harbor a preferred residue at one anchor position and a tolerated residue at the other. The HLA-binding affinity can then be enhanced by a single amino acid substitution to replace the tolerated residue, thereby "fixing" the anchor. Residues at other positions of the peptide can contribute to HLA binding, albeit more weakly, and are referred to as secondary anchor positions *(5)*. These positions can also be substituted to enhance HLA binding of a peptide.

Unlike receptor:ligand interactions that directly result in biological functions such as signal transduction, binding of a vaccine-delivered peptide to an HLA molecule has no direct biological effect. The immunological and pharmacological importance of enhancing this interaction for vaccine design lies in the observation that the affinity of an HLA–peptide interaction directly correlates with immunogenicity *(6)*. By designing epitopes that increase the stability of HLA–peptide complexes on the surface of APCs, the opportunity to effectively engage the cognate T-cell receptor (TCR) and induce a therapeutic T-cell response is enhanced. In addition, it has been hypothesized that CTL specific for a low-affinity, wild-type epitope may be less tolerized than those specific for high-affinity peptides, due to the lower amounts of cell-surface epitope–HLA complexes generated by normal processing and presentation *(7)*. Therefore, by utilizing fixed-anchor analog epitopes as high-affinity immunogens, a vaccine can be used to stimulate these potentially more reactive T-cell populations.

The validity of this approach for generating immunogens with enhanced potency was initially demonstrated in in vitro studies using fixed-anchor analogs derived from HER-2/neu and gp100, where analogs were demonstrated to be more effective for CTL induc-

tion than the wild-type peptides from which they were derived. Single amino acid substitutions to introduce optimal residues at the anchor positions of three peptides derived from the melanoma-associated antigen gp100 were demonstrated to yield analog peptides with enhanced HLA binding and immunogenicity relative to the native sequence *(8)*. In another early study, single amino acid substitutions were made at secondary anchor positions of HER-2/neu-derived peptides resulting in analog epitopes exhibiting enhanced HLA binding and immunogenicity *(9)*.

Our group has utilized HLA motifs and epitope prediction algorithms in combination with high-throughput HLA binding assays to identify novel fixed-anchor analog epitopes from CEA, p53, HER2/neu, and MAGE2/3, five TAAs that are frequently expressed in epithelial-derived tumors *(2,10)*. Initial studies focused on epitopes restricted by the HLA-A2 supertype, and included the identification of wild-type epitopes in addition to fixed-anchor epitopes. From the primary sequences of the four TAAs, approx 1650 motif-positive sequences ranging in size between 8 and 11 residues and carrying the general extended motif that has been associated with the capacity of peptides to bind HLA2.1 were identified. From these motif-positive peptides, a more refined algorithm that takes into account the influence of secondary anchors was used to select 223 peptides for further analysis. Utilizing the HLA-binding assays and this set of peptides, 82 different wild-type epitopes were demonstrated to bind HLA-A2.1 with an IC_{50} of ≤500 n*M*, an affinity previously shown to correlate with peptide epitope immunogenicity *(6)*. Importantly, 123 fixed-anchor analogs with optimized HLA-binding capacity were also generated. From these A2.1 binding peptides, 115 were also demonstrated to bind at least one, and most often two or three additional HLA molecules from the HLA-A2 supertype. Of these HLA-A2 supertype epitopes, 22 wild-type epitopes and 21 analogs were tested for immunogenicity in CTL induction assays using normal donor peripheral blood mononuclear cells (PBMCs). As a read-out, immunoassays measuring cytolysis and/or interferon-γ production were utilized. The CTL cultures demonstrated to be positive for recognition of the immunizing peptide were then further expanded and tested for recognition of naturally processed epitope as presented by HLA- and TAA-matched tumor cell lines. The specificity of the CTL induced by in vitro immunization with peptides was also demonstrated by cold target inhibition experiments for selected epitopes *(2,10)*. For the wild-type peptides, 20 out of 22 (91%) were immunogenic for PBMC from at least one donor, and recognition of tumor cell lines expressing naturally processed antigens was noted for 16 out of 20 (65%) of these epitopes. Interestingly, no significant difference in "hit rate" was observed between MAGE, p53, CEA, and HER2/neu antigens, suggesting that for these TAAs the degree of peripheral tolerance in the CTL compartment is indistinguishable, despite the significantly different protein expression patterns reported for these TAAs.

Fixed-anchor analog epitopes substituted at one or both anchor positions were also evaluated for immunogenicity. Of the analog epitopes harboring a single primary anchor substitution, 100% of the analogs induced CTL when measured against target cells pulsed with the analog peptide. However, to be useful components of a cancer vaccine, epitope analogs must induce CTLs that recognize the wild-type peptide presented on the surface of tumor cells. Recognition of naturally processed antigen on tumor cell lines was demonstrated for 46% (6/13) of the fixed-anchor analogs, thereby validating their use as vaccine immunogens. Analog peptides substituted at both primary anchor positions were also found to be immunogenic, but the CTLs induced were less frequently associated with

recognition of endogenous antigens. These data underscore the importance of using rational design strategies that introduce minimal changes to the epitope, and serve as a cautionary note regarding the use of analog design approaches that yield multiply-substituted analogs.

It is interesting to consider that in general, for both wild-type and analog peptides, the induction of CTLs that are capable of recognizing tumor cell lines as targets is associated with high HLA-binding affinity *(2)*. This conclusion is consistent with previous work by our group and others *(4,8,10)*, although contrary to the postulate that CTL recognizing high-affinity binding epitopes are preferentially deleted from the repertoire, and that epitopes that can induce CTLs recognizing naturally processed epitopes are mostly directed against low-binding-affinity peptides *(11)*.

Several other groups have recently identified HLA-A2.1-restricted fixed-anchor analog epitopes derived from other important TAA. Two analog epitopes derived from an overlapping region of the melanoma differentiation antigen MART-1 have been reported *(12)*. In addition, fixed-anchor analogs derived from NY-ESO-1 *(13)* and Ep-CAM *(14)* have been identified. For each of these analog epitopes, superior immunogenicity relative to the cognate wild-type sequence was demonstrated using human PBMC in an in vitro CTL induction system.

One fixed-anchor analog tested in clinical studies is the gp100.209 (210 M) epitope, which has been utilized as a synthetic peptide delivered to melanoma patients. This analog epitope was reported to be markedly more effective at in vivo CTL induction than was the gp100.209 wild-type peptide when each was administered separately to melanoma patients as a synthetic peptide in Montanide® ISA 51 adjuvant in conjunction with IL-2 *(3)*. In this study, the analog and wild-type peptides induced wild-type peptide-specific CTL responses in 10/11 and 2/8 patients, respectively. Although not a direct comparison of wild-type and analog peptides, two other clinical studies by Weber and colleagues *(15)* and Banchereau and colleagues *(16)* have also demonstrated the immunogenicity of the gp100.209 (210 M) analog, with the latter reporting vaccine-induced CTL recall responses in 12/16 patients. Detailed analyses of the CTL responses induced by this fixed-anchor analog epitope have been conducted *(17,18)*.

All of the fixed-anchor epitope analogs reported to date are restricted by the HLA-A2 supertype. Although this HLA supertype is relatively frequently expressed in patients, 45% on average, vaccines produced using HLA-A2 epitopes are inherently limited in applicability. To expand the application of this rational vaccine design approach, our group is using the same strategy described above to identify CEA-, p53-, HER-2/neu-, and MAGE 2/3-derived fixed-anchor analog epitopes that are restricted by three other common HLA supertypes, -A1, -A3, and -A24 *(19)*. The design of vaccines that combine epitopes restricted by these four HLA supertypes will provide essentially complete population coverage for all patients, regardless of ethnic background.

3. HETEROCLITIC ANALOGS

A second epitope modification strategy involves the introduction of selected single amino acid substitutions at selected positions other than the main HLA anchors of the peptide. The resulting peptides, referred to as heteroclitic analog epitopes, are capable of stimulating unexpectedly potent T-cell responses. A number of different reports have illustrated that heteroclitic analogs are associated with T-cell hyperstimulation, and that

this more potent response is, in fact, mediated by increased binding of the peptide-HLA complex to the TCR *(20,21)*. Importantly for cancer vaccine development, heteroclitic analogs have also been associated with a striking capacity to break tolerance, as shown in a variety of different studies *(22,23)*. Schlom and colleagues identified the first HLA-restricted heteroclitic analog epitope derived from a clinically important human TAA. This modified CEA peptide, designated CAP1-6D, harbors a single substitution of aspartic acid for asparagine at position 6 of the nine amino acid sequence *(24)*. This substitution did not increase the HLA-A2 binding affinity of the peptide, but did result in the hyperstimulation of wild-type specific CTL when the analog peptide was presented on target cells. Importantly, under in vitro conditions where the wild-type peptide was not immunogenic, the CAP1-6D was demonstrated to induce human CTLs capable of recognizing HLA-A2$^+$, CEA$^+$ tumor cell lines. These data provided support for the use of CAP1-6D in human vaccine design (*see* later discussion), and more generally supported the concept of identification and use of heteroclitic analogs derived from human TAA.

For our initial studies to develop and characterize heteroclitic analogs *(23)*, three wild-type, TAA-derived, HLA-A2 epitopes identified in the screening studies described earlier were selected as targets: MAGE-3.112, CEA.691, HER2/neu.157. For each epitope, conserved, semiconserved, and nonconserved substitutions were introduced at all positions in the peptide excluding the MHC anchor positions. These analogs, a total of approximately 350, were screened for heteroclicity by performing antigenicity and dose titration analyses with a CTL line specific for the cognate wild-type peptide. The magnitude of response and shifts in dose responses induced by each analog relative to the wild-type peptide were evaluated, and striking increases of the order of 6–7 logs were detected for some substituted peptides. Importantly for use of these epitopes in vaccine development, the human CTLs generated by in vitro immunization with heteroclitic analogs were able to recognize naturally processed wild-type epitope expressed on tumor cell lines, and were of higher avidity than CTLs induced with the parent peptide. Also, as compared to the wild-type parent peptide, heteroclitic analogs were found to more consistently induce CTLs in vitro. Further, a model heteroclitic analog epitope derived from the murine p53 epitope using this same analog identification strategy was demonstrated to break tolerance to "self" and activate CTLs that recognize tumor cell lines when utilized for in vivo immunization of HLA-A2/K^b transgenic mice.

From these studies, novel heteroclitic analog epitopes were identified, and clinical studies utilizing a subset of these heteroclitic epitopes are currently planned. In addition, several observations relating to the nature and function of epitopes displaying heteroclicity were made. First, it was noted that the amino acid substitutions generally associated with heteroclicity were conservative or semiconservative and occurred in the middle of the peptide at position 3, 5, or 7. Since odd-numbered positions in the middle of an HLA-bound peptide may be pointing up toward the T-cell receptor, this observation is consistent with the effect being mediated by an increased interaction with TCRs. Some previous studies implied that modulation of T-cell responses by heteroclitic analogs involves modification of peptide residues that directly contact the TCRs *(20,24)*, but this finding was not corroborated by our study *(23)*, which indicated that heteroclicity is likely to be generated by subtle alterations in epitope conformation rather than by gross alterations of TCR contacts or MHC binding capacity. Salazar et al. have reported increased phosphorylation of Zap-70 in heteroclitic analog-stimulated T cells *(22)*, but the structural and signaling mechanisms involved in heteroclicity require further investigation.

From a practical standpoint, our studies have important implications for vaccine development. By identifying the substitution patterns most frequently associated with heteroclicity, "rules" were developed that can streamline the analog screening process. We have found that we can successfully identify heteroclitic analogs from all epitopes, and our success rate of analog prediction is in the 20–40% range, which represents about a 20-fold increase over random prediction. By combining this targeted substitution strategy with high-throughput screening, heteroclitic analog epitopes derived from any TAA and restricted by commonly expressed HLA supertypes can be identified and utilized for cancer vaccine design.

The HLA-A2 restricted, CEA-derived CAP1-6D heteroclitic analog identified by Schlom and colleagues *(24)* has been utilized in human clinical trials. In a study by Fong and colleagues, the CAP1-6D peptide was demonstrated to effectively expand epitope specific human CTL when delivered using dendritic cells and measured using tetrameric HLA/peptide complexes (tetramers) *(25)*. These CTL expansion data, and to a lesser extent the ELISPOT data reported, correlated with the observed clinical responses. Previous clinical studies using the wild-type CAP-1 peptide had demonstrated relatively weak immunogenicity *(26)*, underscoring the potential significance of these early results obtained using the CAP1-6D heteroclitic analog. Additional clinical studies testing the CAP1-6D epitope and the novel heteroclitic epitopes described above should provide the human immunological data needed to address the general applicability of this approach for cancer vaccine design.

4. SUMMARY

The rational design of analog epitopes with enhanced immunogenicity is a promising strategy for improving on the cancer vaccine clinical outcomes to date. The use of fixed-anchor and heteroclitic analog epitopes is compatible with most all delivery systems, and these modified antigens should expand the quality and breadth of antitumor T-cell responses achievable in humans by facilitating the stimulation of CTLs specific for epitopes that in their natural form are too weakly immunogenic to be effective vaccine components. In addition, these optimized immunogens should complement other approaches directed at enhancing T-cell induction, maintenance, and effector function *(1)*.

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43

Transplantation immunology

J. Andrew Bradley

An understanding of relevant transplant immunology is an essential requirement for surgeons involved in the care of patients undergoing organ transplantation. Aspects of the subject are also likely to be of general relevance in the wider field of surgery.

It is now over half a century since the pioneering studies of Peter Medawar firmly established that tissue grafts are rejected because of the specific immunological response they provoke and not, as many had previously thought, because of a non-specific inflammatory response. Since this landmark observation, remarkable progress has been made in our understanding of graft rejection and in how best to try and overcome it. To those readers not familiar with the specialty of transplantation, the prospect of transplant immunology can seem somewhat daunting and the apparent complexity of the terminology used may do little to allay this view. The reader should not be deterred. The key concepts in transplant immunology are not very difficult to grasp and the importance of the science in underpinning progress in the clinic cannot be overstated. This chapter, written with the generalist in mind, outlines the immunopathology of graft rejection and the role of the histocompatibility laboratory in transplantation. The different immunosuppressive agents and regimens used to prevent allograft rejection are described along with the complications of non-specific immunosuppression, namely opportunistic infection and malignancy.

TERMS AND DEFINITIONS

To aid the reader, some of the important terms used extensively in transplantation immunology are defined below.

An *allograft* is a graft between two genetically dissimilar individuals of the same species (the old term *homograft* is no longer used). *Allogeneic* cells or tissue are genetically dissimilar but of the same species.

A *syngeneic* graft (or *isograft*) is a graft between two genetically identical individuals, as in the case of identical twins. An *autograft* is a graft originating from and applied to the same individual as in the case of a skin graft.

A *xenograft* is a graft between two different species.

Human leucocyte antigens (HLA or HLA antigens) comprise two classes (class I and class II) of cell surface proteins whose principal function is to display peptide antigens so they can be recognised by T cells. In the context of transplantation they are the principal transplant antigens or *alloantigens*.

The *major histocompatibility complex (MHC)* is a region of the mammalian genome (designated the HLA region in humans) encoding for proteins that have an important role in immunity. These include HLA antigens in the human.

Polymorphism refers to differences in a particular gene (*genetic polymorphism*) or the amino acid sequence of a protein, e.g. *HLA polymorphism* between different individuals.

ALLOGRAFT REJECTION

Organ and tissue allografts provoke a very strong immunological response and, unless potent immunosuppressive therapy is given, rapid and complete graft destruction within one or two weeks is usually inevitable. The immune response to an allograft is directed against so-called transplant antigens (histocompatibility antigens) expressed on the surface of cells within the graft. Graft rejection is an adaptive rather than an innate or non-specific immune response and experiments where grafts are performed between different inbred strains of mice show that it has both antigen specificity and immunological memory – the two cardinal features of an adoptive immune response (Figure 43.1). The antigen specificity of rejection is provided by T and B lymphocytes that recognise transplant antigens by their antigen-specific cell surface receptors.

Transplantation is not a natural phenomenon and no special immunological effector mechanisms exist solely to prevent successful transplantation. Graft rejection is mediated instead by the cellular and humoral effector mechanisms that have evolved naturally to provide protection against invading pathogens. Allograft rejection is a T cell-dependent immune response and cytotoxic T cells, delayed-type hypersensitivity (DTH) responses and T cell-dependent antibody responses may all contribute to the rejection process.

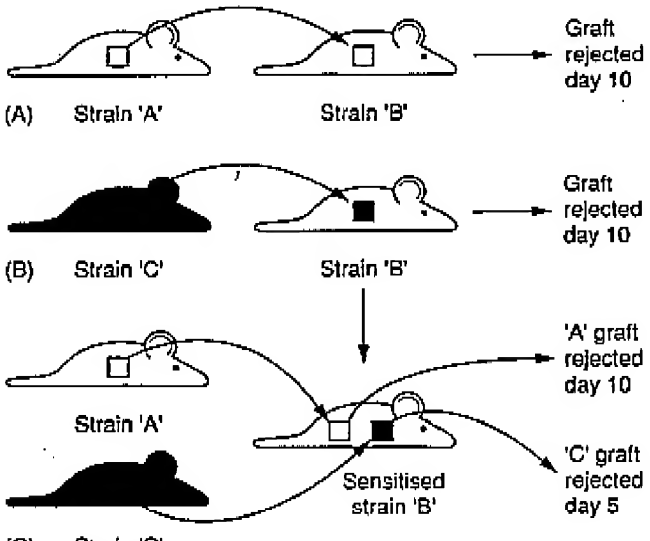


Figure 43.1 Graft rejection is due to an adaptive immune response. **A,B.** Inbred 'B' strain mice reject 'A' strain and 'C' strain skin grafts by 10 days. **C.** When 'B' strain mice that have been sensitised by a previous 'C' strain skin graft are challenged with second grafts they reject 'C' strain but not 'A' strain grafts more rapidly. Graft rejection therefore shows both antigen specificity and memory, the two cardinal features of an adaptive immune response. Adoptive transfer of T lymphocytes alone can transfer accelerated donor specific rejection from a sensitised to a naive recipient.

The ABO blood group barrier

The A, B, O blood group antigens are a crucial consideration in organ transplantation. These antigens result from structural polymorphisms in carbohydrate residues on glycolipids expressed on the surface of red blood cells. Individuals who do not possess a particular blood group antigen develop cross-reactive antibodies against that antigen through exposure of lymphocytes to normal intestinal bacteria bearing similar or cross-reactive antigens. Because blood group antigens are also expressed on the vascular endothelium of organs, it is essential to ensure that allografts are ABO blood group compatible otherwise preformed antibodies are likely to result in hyperacute rejection. Blood group O individuals have naturally occurring antibodies to blood group A and group B antigens and can only be given grafts from blood group O donors (the universal donor). Blood group A recipients can be given grafts from blood group O or A donors. Conversely, blood group B recipients can receive grafts from blood group O and B donors. Recipients who are blood group AB have no ABO antibodies and are universal recipients. Although organ allografts must be ABO compatible there is no need to ensure compatibility for Rhesus blood group and it is not considered when allocating organs for transplantation.

The MHC

Any protein that differs in amino acid composition between donor and recipient may, in principle, act as a transplant antigen. Assuming blood group compatibility, by far the most important transplant antigens are those encoded by genes in two regions (the class I and class II regions) of a segment of the genome designated the major histocompatibility complex (MHC). The major histocompatibility genes that are located collectively in this region of the mammalian genome were first identified in rodents through their influence in controlling acute rejection of skin grafts – hence the designation of the region as the major histocompatibility complex. The MHC did not, of course, evolve as a barrier to transplantation since this is an entirely non-physiological situation created by transplant surgery. The role that products of the MHC play as transplant antigens is instead a byproduct of their fundamental physiological function as immune recognition elements.

Membrane glycoproteins encoded by genes in the class I and class II regions of the MHC act like a scaffold to selectively display foreign antigens as linear peptide fragments at the cell surface for surveillance by T lymphocytes. This, coupled with the fact that MHC molecules are highly polymorphic and abundantly expressed on the surface of most cell types, explains their unique potency as transplant antigens. The extensive polymorphism of MHC molecules between individuals is unfortunate from the perspective of organ transplantation because it ensures immunological incompatibility unless the prospective recipient and donor are genetically identical. However, it is clearly of great biological advantage for the species as a whole because it maximises the chance that there will always be individuals able to mount an effective immune response to new and potentially dangerous pathogens that might otherwise threaten the entire species.

HLA

In humans the major histocompatibility antigens were first identified using serological techniques to detect their presence on leucocytes. This led to their designation as human leucocyte antigens, abbreviated to HLA, and the same term is used to describe the human MHC. The HLA complex is the most polymorphic region of the human genome and was the first region to be sequenced in detail. It spans around 4000 kilobases of DNA and is located on the short arm of chromosome 6. The genes within the HLA are divided into three regions, known as class I, class II and class III (Figure 43.2). Genes in the class I region encode, in order of discovery, HLA-A, HLA-B and HLA-C (the so-called classic class I MHC antigens) along with other non-classic class I molecules such as HLA-E, HLA-F and HLA-G. Genetic loci in the class II region encode the class II antigens HLA-DR, HLA-DP and HLA-DQ, along with other proteins that are involved in antigen processing but do not act as transplant antigens. The class III region is situated between the class I and class II regions and encodes a number of proteins of importance in immunity although none are major transplant antigens. The most important transplant antigens are all encoded by distinct genes in the class I and class II regions. The class I region gene products of most relevance to organ transplantation are HLA-A and HLA-B (in order of discovery) and the most important class II region product is HLA-DR (for D Related). These three loci or their products are those that are routinely typed to determine the tissue match before organ transplantation.

Inheritance of HLA and HLA type

The HLA genotype or HLA profile of an individual is determined by the particular combination of HLA alleles (different forms of

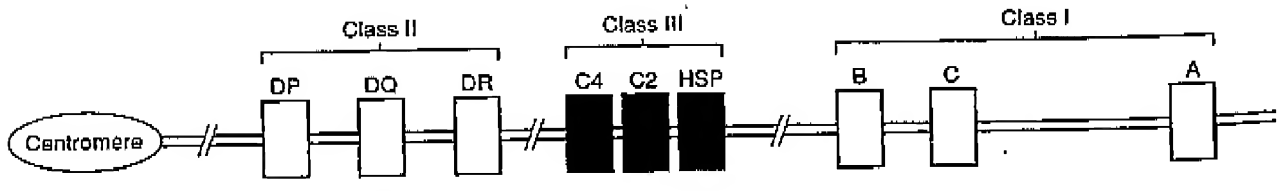


Figure 43.2 Organisation of HLA on the short arm of chromosome 6.

the same gene) that they have inherited from each of their parents. HLA genes are co-dominantly expressed so that, depending on whether an individual inherits the same or a different HLA gene polymorphism from each parent, they will express one or two different isoforms of each HLA molecule. During meiosis the HLA genes of a particular chromosome remain closely linked together so that the HLA haplotype inherited from each parent is generally inherited as a complete unit. HLA haplotypes are inherited according to simple Mendelian genetics. As shown in Figure 43.3, there is a one in four chance that two siblings will share the same parental haplotypes, or that they will share neither HLA haplotype, and a one in two chance they will share one HLA haplotype.

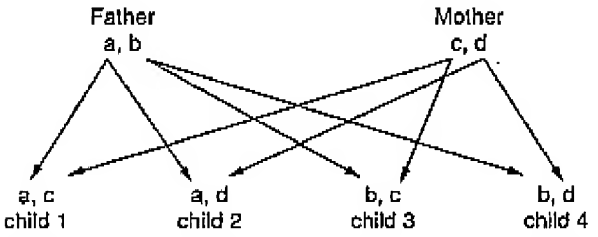


Figure 43.3 Inheritance of HLA. Each child inherits one HLA haplotype (designated a–d) from each parent. In the example shown, child 1 shares one HLA haplotype (i.e. is haploidentical) with child 2 and child 3 but does not share either haplotype with child 4.

As already noted, in clinical organ transplantation the HLA molecules of most importance are HLA-A, HLA-B and HLA-DR. Each chromosome 6 encodes a single HLA-A, -B and -DR antigen. An individual may therefore express between three and six different isoforms of these HLA antigens depending on whether they are homozygous or heterozygous at each of the three loci. The situation for HLA-DR is sometimes rather more complex because chromosome 6 occasionally carries an additional HLA-DR gene so an individual could express up to four different HLA-DR molecules.

Occasionally, a parental HLA haplotype is not inherited intact and crossover and recombination of DNA occur so that part of one haplotype recombines with part of another haplotype, giving rise to a new combination (or allele) of HLA genes. Genetic recombination of HLA is not entirely random in nature and certain combinations of HLA genes are found associated with each other more frequently than would be predicted. This non-random gene association of HLA genes is known as linkage disequilibrium and sometimes extends to an entire HLA haplotype. It probably arises because the resulting combination of genes making up the haplotypes confers a survival advantage by providing better protection against pathogens present in the geographical environment of a particular population or ethnic group.

HLA polymorphism

The MHC is the most polymorphic region of the human genome and there is extensive polymorphism of both HLA class I and class II molecules. Much of the genetic polymorphism observed encodes for amino acids situated in the regions of the HLA molecule that form the peptide binding groove and that bind with the T cell receptor. The number of amino acid differences between different HLA allotypes varies between one and 50 but even very small differences in amino acid sequence are sufficient to create a strong histocompatibility barrier to transplantation.

Before molecular typing techniques became available, HLA typing was performed by serological techniques. As new allelic variants were discovered and their serological specificity defined, they were assigned numerically under the letter designating their allele. In many cases the original antigen was subsequently *split* as further serological analysis revealed further variants and the nomenclature became increasingly complex. Using carefully defined panels of sera from individuals who had been sensitised to HLA molecules, around 20 different alleles at the HLA-A, 40 at the HLA-B and 20 at the HLA-DR locus were identified. DNA analysis has revealed that the true number of functional allelic variants is several times greater than that revealed by serological analysis, with over 100 HLA-A alleles, 200 HLA-B alleles and 200 HLA-DR alleles.

To standardise the designation of HLA genes and their protein products, a new nomenclature was introduced under the auspices of the World Health Organisation (WHO). In this system, all HLA allotypes are classified on the basis of their DNA sequence. Individual HLA alleles are identified first by their gene locus followed by a four-digit number, with the first two digits signifying the HLA specificity and the next two digits denoting the HLA subtype. Under the WHO nomenclature, for example, all alleles of the HLA-A gene are given the prefix A* followed by the digits 01 for alleles encoding the serologically defined A1 antigen, 02 for alleles encoding A2, etc. The two individual alleles of HLA-A1 are designated A*0101 and A*0102 and the numerous alleles of HLA-A2 are designated A*0201 to A*0226 respectively. Additional digits are used to indicate DNA polymorphisms that do not result in functional allelic variants and the suffix N is used to denote null alleles where a particular polymorphism prevents gene expression.

Structure and function of HLA class I and class II molecules

HLA molecules are of such fundamental importance to graft rejection that a full description of their structure and function is merited. The physiological function of HLA class I and class II molecules is to bind foreign antigen in the form of linear peptide and display it at the cell surface for surveillance by T cells. Antigen displayed in this form is recognised by antigen-specific T lymphocytes and these then trigger a protective cellular immune response. The role of HLA molecules in this regard is crucial since the antigen receptor on T lymphocytes cannot recognise proteins from pathogens in their native form but only after they have been broken down and displayed as peptides bound to HLA class I or

class II molecules. Moreover, the specificity of the T cell receptor is such that it cannot recognise peptide *per se* but instead recognises a molecular composite comprising the antigenic peptide along with adjacent parts of the HLA molecule to which it is bound.

The three-dimensional structure of class I and class II molecules is broadly similar in that they both possess a deep peptide-binding groove walled by two parallel alpha helices and floored by a beta-pleated sheet (Figure 43.4). This functional structure is achieved in a different way by the two classes of HLA molecule. Class I HLA molecules are made up of a transmembrane heavy chain (the alpha chain) associated non-covalently with a small non-polymorphic molecule called beta-2 microglobulin that is encoded by chromosome 15. The alpha chain of class I has three extracellular immunoglobulin-like domains and two of these (the alpha-1 and alpha-2 domains) make up the peptide-binding groove. Class II HLA molecules, on the other hand, comprise two membrane bound chains, the alpha and the beta chains, each of which has two extracellular immunoglobulin-like domains. The peptide-binding groove is formed by the alpha-1 domain of the alpha chain and the beta-1 domain of the beta chain.

Peptides can only bind within the groove of a MHC molecule if they contain the relevant amino acids, or *anchor residues*, which collectively constitute a peptide-binding motif for that particular HLA molecule. The lengths of peptides that can be accommodated in HLA molecules are also constrained by the architecture of the peptide-binding groove and this differs for HLA class I and class II. HLA class I molecules have a binding groove that is closed at each end and bound peptides are usually 8–10 amino acids in length. The peptide-binding groove in class II molecules is open ended, allowing longer peptides, typically 12–25 amino acids in length, to be accommodated.

An important point to appreciate, particularly in the context of transplantation, is that cell surface HLA molecules rarely have 'empty' peptide-binding grooves. When they are not occupied by an antigenic peptide derived from pathogens, they are filled instead by non-antigenic peptides derived from intracellular and cell surface proteins. In fact, even in the presence of an infection, the majority of bound peptides in HLA are still derived from self-proteins rather than from the invading microorganism.

Peptides presented by the two classes of HLA molecule are derived from two different antigen-processing and presentation pathways (Figure 43.5). HLA class I molecules present peptides derived from the intracellular environment. Endogenous proteins synthesised in the cytosol (self-proteins, viral proteins and intracytoplasmic bacteria) are broken down into peptides and transported to the endoplasmic reticulum where they bind to HLA class I molecules and are then transported to the cell surface. HLA class

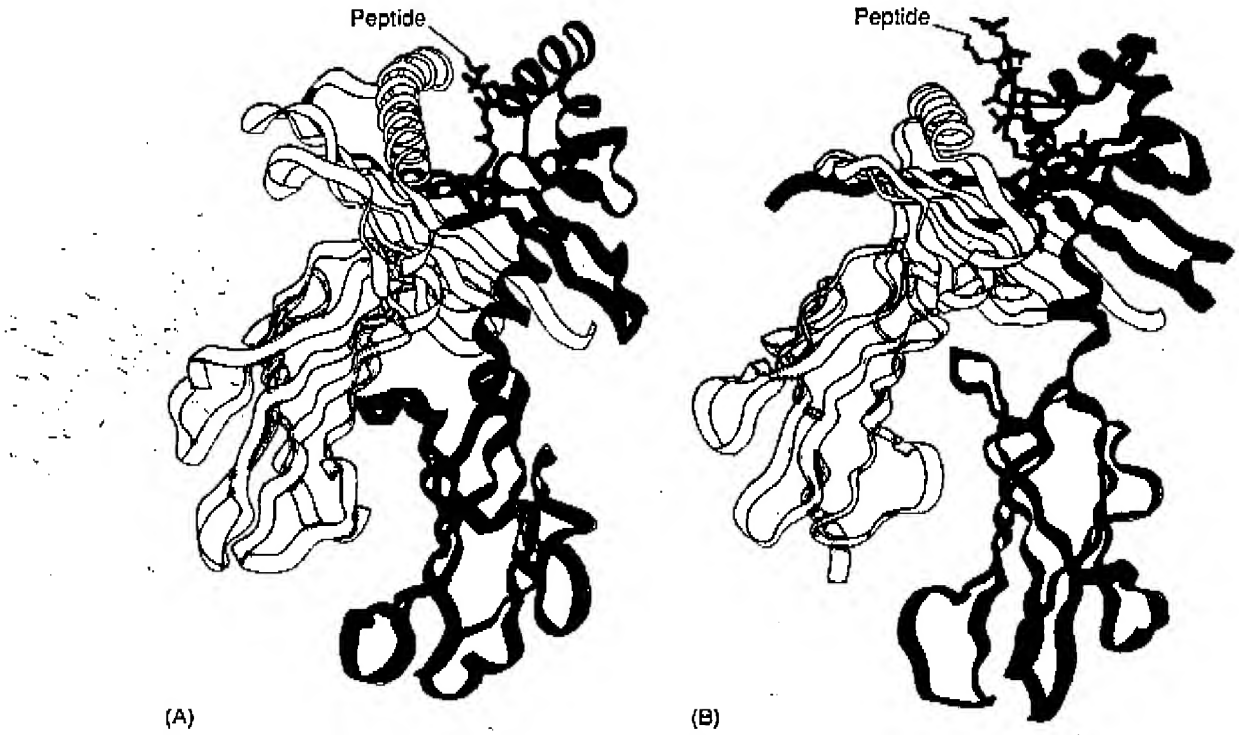
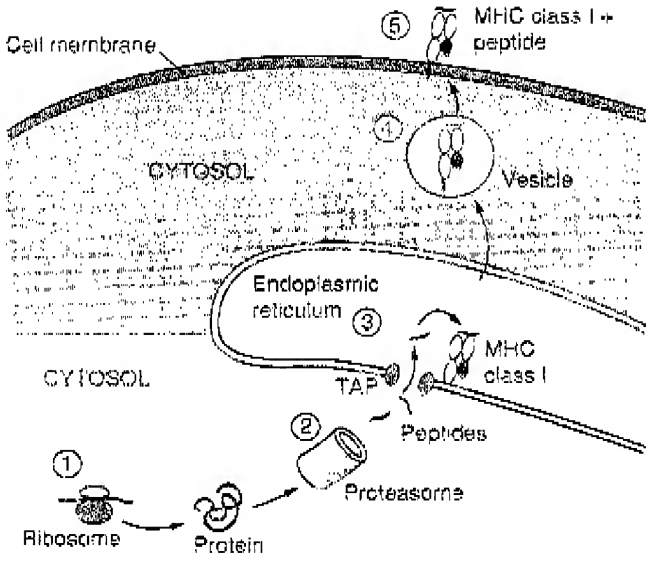
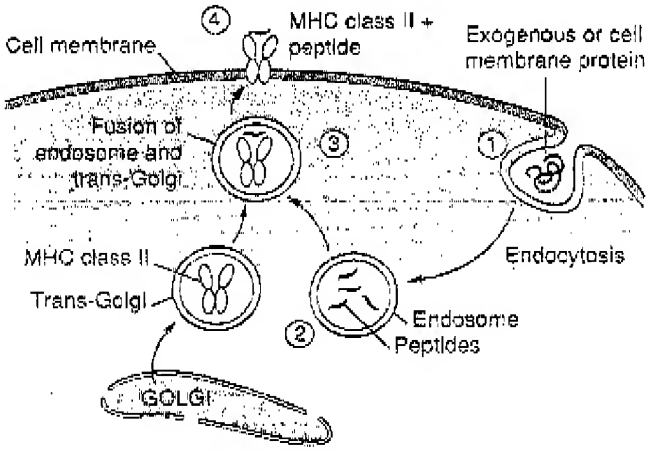


Figure 43.4 Structure of HLA class I (A) and HLA class II (B). HLA class I and class II molecules have a similar three-dimensional structure. The membrane distal domains of the molecules form a cleft in which is bound an antigenic peptide for recognition by a T lymphocyte. Redrawn with permission from Stern and Wiley, *Structure* 1994; 2:243–252.



(A) Class I MHC pathway



(B) Class II MHC pathway

Figure 43.5 Antigen processing and presentation. **A.** MHC class I molecules present peptides derived from intracellular proteins. The proteins are broken down into peptides by the proteasome and then transported to the endoplasmic reticulum by the ATP-dependent TAP transporter where they are loaded into class I molecules before transfer to the cell surface in a vesicle. **B.** MHC class II molecules present peptides derived from proteins at the cell surface or from the extracellular environment. After endocytosis, the protein is broken down into peptides within the endosome. The endosome then fuses with a trans-Golgi containing class II molecule and the class II MHC/peptide complexes are transported to the cell surface.

II molecules, on the other hand, present peptides derived from the extracellular environment. Cell surface molecules (including HLA molecules themselves) and extracellular proteins (such as bacterial proteins) are taken up within endosomes or lysosomes. There they are degraded by proteases into peptides before fusion of the endocytic vesicle with the trans-Golgi network results in loading of the peptides into class II molecules and transport back to the cell surface.

There are important differences in the cellular distribution of HLA class I and class II molecules. HLA class I is expressed on the surface of nearly all nucleated cell types. HLA class II, in contrast, has a more restricted cell distribution and is expressed most abundantly on cells of the immune system that have a special role in antigen presentation, notably dendritic cells, macrophages and B lymphocytes, the so-called *professional antigen presenting cells*. Some other cell types, such as the vascular endothelium and parenchymal cells of an organ graft, can also express HLA class II and such expression is upregulated during inflammatory responses, such as allograft rejection, by the proinflammatory cytokine interferon-γ.

Alloantigen recognition by T cells

The initial cellular interaction that triggers graft rejection is the cognate interaction of alloantigen on the surface of an antigen-presenting cell with an antigen-specific T cell through its T cell receptor (TCR) (Figure 43.6). The TCR is a membrane-bound immunoglobulin-like molecule comprising two similar polypeptide chains (designated alpha and beta). Each individual T cell clone expresses a unique TCR with particular antigen specificity and all the TCRs on a given T cell have the same antigen specificity. Although the TCR molecule itself lacks a full cytoplasmic tail and cannot deliver an intracellular signal, it is closely associated at the cell surface with a series of non-polymorphic proteins called the CD3 complex and these are responsible for intracellular signalling following engagement of the TCR with antigen. Together, the TCR and the CD3 complex form a functional unit for antigen recognition called the TCR complex. Mature T cells

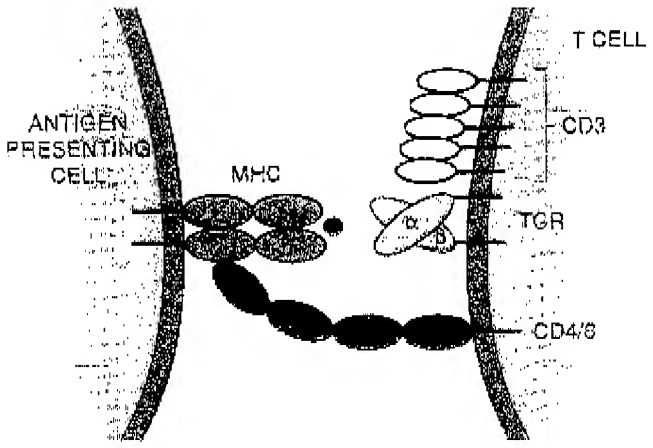


Figure 43.6 Interaction of the T cell receptor complex with MHC/peptide complex. The T cell receptor complex comprises the TCR, the CD3 complex and either CD4 or CD8. The CD3 complex transduces the intracellular signal from the TCR. CD8 and CD4 bind to non-polymorphic regions of the class I and class II MHC respectively.

can be divided into two subclasses according to whether they express CD4 or CD8 cell surface glycoprotein molecules. Whereas CD8 T cells are cytotoxic and able to kill target cells bearing antigen, CD4 T cells have a helper function and are known as helper T cells. The non-polymorphic CD8 and CD4 molecules function as co-receptors during antigen recognition by binding to non-polymorphic regions of HLA molecules on the antigen-presenting cell. Because CD8 binds only to HLA class I and CD4 only to HLA class II, the two functionally distinct T cell subsets recognise peptide antigens derived from different antigen-processing pathways.

The ligation of the TCR complex by HLA-antigen leads to activation of ZAP-70, a cytoplasmic tyrosine kinase molecule. Activation of ZAP-70 initiates a series of complex downstream signalling cascades that culminate in the activation of a set of transcription factors comprising NFκB, NFAT and AP1. These three transcription factors then turn on the genes necessary for T cell activation and differentiation. However, the signals arising from the TCR complex are not by themselves sufficient to cause full T cell activation. Indeed, ligation of the TCR alone (so-called signal 1) may encourage the T cell to become unresponsive or anergic instead of fully activated. To achieve full activation, the T cell also requires the delivery of additional or co-stimulatory signals (collectively comprising signal 2). These are provided when non-polymorphic receptor molecules on the T cell surface bind to their ligands on the surface of the antigen-presenting cell and provide supplementary activation of ZAP-70. Important co-stimulatory molecules expressed on antigen-presenting cells include CD80 and CD86, that both bind to CD28, and CD40 whose ligand is CD154.

Allorecognition pathways

HLA antigens in a transplanted organ can be recognised by two different routes designated the direct and indirect pathways of allorecognition. Until recently, the direct pathway of allorecognition was regarded as the principal route for allorecognition but it has become increasingly clear that indirect allorecognition is also important.

Direct allorecognition

During direct allorecognition, which is unique to transplantation, the recipient T cell recognises intact donor HLA molecules complexed with endogenously derived peptide expressed on the surface of a donor strain antigen-presenting cell (Figure 43.7). The most effective type of donor strain antigen-presenting cell (APC) is the interstitial dendritic cell, known, in the context of transplantation, as a passenger leucocyte. The interstitial dendritic cell is a bone marrow-derived leucocyte that is widely distributed throughout all of the commonly transplanted organs. These cells are richly endowed with both class I and class II HLA molecules and are armed with the full complement of co-stimulatory molecules needed to cause T cell activation.

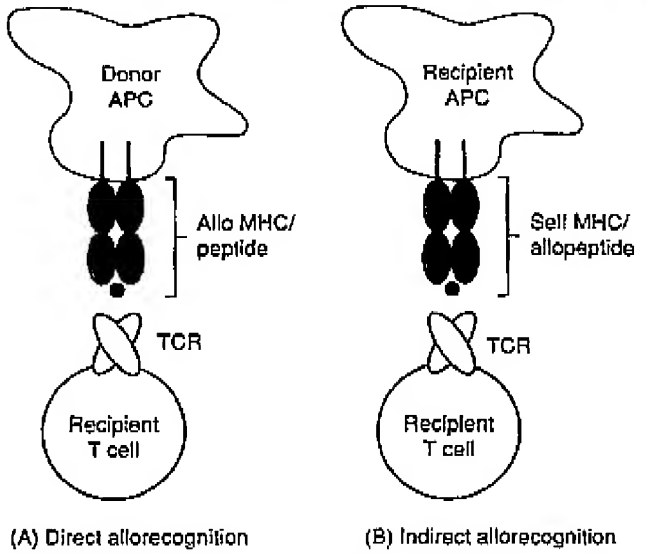


Figure 43.7 Alloantigen recognition pathways. A. In direct allorecognition, the recipient T cell recognises allogeneic MHC (plus bound endogenous peptide) on the surface of a donor APC. B. In indirect allorecognition the recipient T cell recognises and responds to shed donor-derived alloantigen after it has been taken up by a recipient APC and presented as peptide bound to self-MHC.

Why should T cells recognise and respond to allogeneic HLA molecules but not self-HLA molecules? To answer this question it is necessary to consider how the normal T cell repertoire is selected as it develops within the thymus gland. As T cells mature they undergo a two-stage selection process. First, they undergo positive selection during which only those T cells that are specific for complexes of self-HLA molecules and peptides derived from breakdown of self-proteins are allowed to survive. All remaining non-reactive T cells are destined to die by apoptosis. Next, the developing T cells undergo a negative selection process during which any T cells whose antigen receptors bind too strongly to self-HLA and self-peptide are deleted. This is an important selection process because if high-affinity autoreactive T cells were not purged in this way autoimmunity would develop. The final T cell repertoire remaining after thymic selection, therefore, comprises T cells with a wide range of antigen specificities, all recognising self-HLA and bound endogenous peptide but with insufficient binding affinity to cause T cell activation. During an immune response to a foreign protein antigen, T cells recognise the complex of antigenic peptide and self-HLA with high affinity and respond accordingly.

When, following organ transplantation, T cells encounter intact allogeneic HLA molecules and their bound endogenous peptides on donor APC, about 5% of them are activated. This is an enormous response considering that only around one in 10000 T cells recognises a foreign protein antigen during a physiological immune response. Two hypotheses have been advanced to explain why so many T cells respond so strongly to intact allogeneic HLA. The 'multiple binary complex hypothesis' states that the responding T cells comprise multiple T cell clones each responding to a

molecular complex of foreign HLA and one of the thousands of different endogenous peptides bound within the groove of the allogeneic HLA molecule. The 'high determinant density hypothesis' states that the specificity of the alloreactive T cells is predominantly directed against the allogeneic HLA molecule and that the identity of the bound peptide is not critical. Accordingly, all of the hundreds or thousands of HLA molecules expressed on an individual APC will be recognised as 'foreign'. This is in marked contrast to a physiological immune response where only a relatively small percentage of self-HLA molecules present a foreign antigenic peptide and the remainder continue to present non-immunogenic self-peptides. The high density of antigenic HLA molecules present during direct allorecognition exceeds the threshold for the number of TCR molecules needed to trigger T cell activation, even when the affinity of the TCR for allogeneic HLA is not very high. These two hypotheses outlined above are not mutually exclusive and a combination of both is the likely explanation for the strength of the direct alloimmune response.

Indirect allorecognition

During the indirect pathway of allorecognition, recipient T cells recognise allogeneic HLA molecules (or any other polymorphic protein unique to the donor) after they have been processed and presented as antigenic peptides by recipient HLA molecules on the surface of a self-APC (see Figure 43.7). The indirect pathway is therefore analogous to the normal immune response to a foreign protein and the donor interstitial cell plays no role other than acting as a further source of allogeneic HLA molecules for uptake and processing by recipient APCs. Within a few days of transplantation, donor interstitial dendritic cells migrate from the donor organ into the recipient lymphoid tissue. This leaves the graft devoid of 'professional' APCs and considerably reduces the scope for further activation of T cells by the direct allorecognition pathway. It has been postulated that once donor dendritic cells have disappeared, the indirect allorecognition pathway assumes an increasingly important role in graft rejection. The indirect allorecognition pathway may be particularly important during chronic allograft rejection. As noted earlier, extracellular protein antigens are taken up by APCs via the endocytic pathway and the processed antigen is then presented to CD4 T cells by HLA class II molecules. HLA antigen shed from donor cells is treated the same way and therefore generates a predominantly CD4 T helper cell response. In contrast to T cells that recognise HLA directly, CD4 T cells activated by the indirect pathway are not able to directly damage the graft since they do not recognise intact donor HLA on target cells. They are able, however, to provide essential T cell help for CD8 T cells activated via the direct pathway. They can also provide cognate help for alloantibody and can mediate an intragraft DTH response.

Minor histocompatibility antigens

Although HLA antigens are the most important barriers to successful transplantation, peptides derived from other polymorphic proteins expressed in the donor can also act as transplant antigens. These antigens, known collectively as minor histocompatibility antigens, are presented by recipient APCs via the indirect pathway of allorecognition. Multiple minor histocompatibility antigens are likely to be present in all organ transplants and one of the best characterised in experimental mouse studies is the male or HY antigen. Although minor histocompatibility antigens are, as their name implies, weak transplant antigens, multiple minor antigens can exert a cumulative effect and they can cause rejection episodes when kidney transplants are performed between HLA identical sibs.

B cell allorecognition

In contrast to T cells that can only recognise HLA molecules as peptide fragments, B lymphocytes are able to recognise, through cell surface immunoglobulin receptors, conformational antigenic epitopes expressed by the intact allogeneic HLA molecule. Alloantibody production by B lymphocytes is critically dependent on the provision of T cell help. The B cell internalises allogeneic HLA molecules by receptor-mediated endocytosis and then processes and presents HLA-derived peptide in the peptide-binding groove of HLA class II on its cell surface. The processed alloantigen is recognised by T cells activated by the indirect pathway and these then deliver the essential help required for B cell activation and alloantibody production. T cell help for B cells takes the form of cognate receptor ligand interactions such as CD40 with CD154 and cytokines such as IL-4, IL-5 and TGF-β. Cytokines are important for promoting B cell maturation into antibody-producing plasma cells and for promoting the production of different antibody classes (IgM to IgG) and subclasses (IgG1, IgG2a and IgG2b). The antibody isotypes retain their original antigen specificity but display different effector functions. As will be outlined later, alloantibodies make an important contribution to graft rejection. They may cause hyperacute rejection of a kidney allograft in a sensitised recipient and they may contribute to both acute and chronic rejection in all types of organ allograft.

Lymphocyte activation, differentiation and expansion

During the first few days after transplantation, donor-derived dendritic cells migrate from the graft into the secondary lymphoid tissue of the recipient. Here they present intact donor alloantigen to naïve alloreactive T cells and B cells. In addition, intact alloantigen is released or shed from the graft as membrane fragments and circulates to the secondary lymphoid tissue where it is taken up by recipient APCs which then activate alloreactive lymphocytes. After T and B cells are activated they undergo a period of clonal expansion and differentiate into regulatory and effector cells. Circulating naïve T and B cells may also first encounter alloantigen within the graft rather than the peripheral lymphoid tissue and then undergo clonal expansion *in situ*. Activated T cells acquire the ability to produce a range of regulatory cytokines,

upregulate or downregulate the expression of various cell surface molecules and develop the capacity to kill target cells by producing lytic granules. B cells differentiate, with the aid of T cells, into antibody-producing plasma cells. The differentiation and clonal expansion of lymphocytes is dependent on a range of cytokines, including IL-2, IL-4, IL-7, IL-9 and IL-15. These cytokines therefore play a critical role in allograft rejection.

Lymphocyte cell surface receptors for the different T cell growth factors comprise units of two or three polypeptide chains. All of the receptors share a common gamma chain. IL-2 and IL-15 are particularly important T cell growth factors and although their receptors each have a unique alpha chain, they also share the same IL-2β chain. Not surprisingly, therefore, IL-2 and IL-15 have a very similar biological function. There is considerable redundancy and functional overlap among all the T cell growth factor family of cytokines and this may explain why targeting an individual cytokine or its receptor (e.g. IL-2/IL-2 receptor) does not necessarily prevent graft rejection.

After activation, T cells may differentiate into at least three functionally distinct populations. CD8 T cells differentiate into cytotoxic effector cells. CD4 T cells, on the other hand, may differentiate into either Th1 or Th2 cells. These two T helper cell subsets are defined in terms of the pattern of cytokines they produce and have a counter-regulatory effect on each other that may polarise towards either a Th1- or a Th2-dominated response. Th1 cells produce IL-2 and IFN-γ and are responsible for directing cell-mediated immune responses whereas Th2 cells secrete IL-4, IL-5, IL-10 and IL-13 and are mainly responsible for promoting alloantibody responses. Through release of cytokines, CD4 T cells play a pivotal role in orchestrating the different cellular and humoral effector mechanisms that contribute to graft rejection (Figure 43.8).

As the alloimmune response develops there is progressive mononuclear cell infiltration and deposition of alloantibody within the graft. In unmodified animal models of transplantation, there is complete graft destruction by 7–10 days. Infiltration of the graft by mononuclear cells starts very soon after transplantation and is facilitated by tissue injury arising during the transplant procedure. An organ graft inevitably suffers a degree of ischaemic injury during transplantation and this contributes to the early inflammatory response. Activated macrophages that have been resident or recently recruited to the graft produce cytokines such as IL-1, IL-6 and IL-8. These cause upregulation of adhesion molecules such as P- and E-selectins, vascular addressins and intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2) on the endothelial cell surface and facilitate binding of lymphocytes that express complementary adhesion molecules. Over the course of the next few days, increasing numbers of lymphocytes home to the graft where, after binding to endothelial cells, they migrate into the graft tissue under the control of chemokines such as IL-8, macrophage inflammatory protein (MIP), RANTES and macrophage chemoattractant protein-1 (MCP-1).

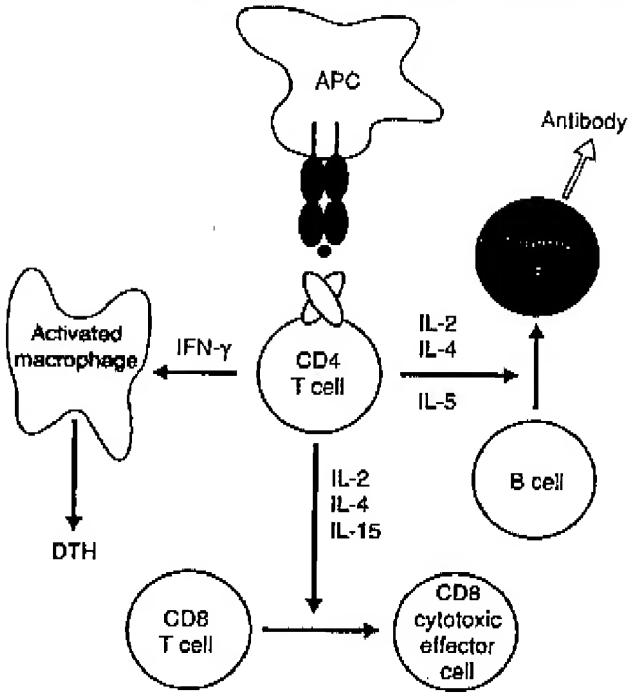


Figure 43.8 Central role of the CD4 T helper cell in orchestrating allograft rejection. After activation the CD4 T cell produces a range of cytokines that promote the development of DTH, CD8 cytotoxic effector cells and antibody.

Intragraft effector mechanisms

Most of the different cellular and humoral effector mechanisms that the immune system has evolved to protect against invading pathogens can play a role in allograft rejection. As already noted, alloantibodies play an important role in tissue destruction, especially in the sensitised recipient. After binding to target antigens within the graft vasculature, antibodies may induce target cell damage by a variety of mechanisms. Bound antibody may activate the complement cascade via the classic pathway, culminating in formation of membrane attack complexes and cell injury or death. Antibody may also cause cell damage indirectly by guiding non-specific effector cells such as macrophages and non-specific cytotoxic cells onto target cells in the graft. Finally, alloantibody may, through cross-linking cell surface molecules on endothelial cells, cause endothelial cell activation leading to release of cytokines and growth factors.

Specific cytotoxic CD8 T cells recognising donor HLA class I antigens are able to kill target cells by release of lytic enzymes such as perforins and granzymes, by triggering the Fas death pathway and through release of the proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α). CD4 and CD8 T cells are able to initiate a DTH response through release of proinflammatory cytokines, such as IFN-γ, leading to recruitment and activation of macrophages. Activated macrophages function as non-specific effector cells by releasing a wide range of noxious

agents. These include proinflammatory cytokines such as TNF-α, enzymes such as lysozyme and hydrolase and oxygen derivatives such as hydrogen peroxide and nitric oxide. Activated eosinophils may also contribute to allograft rejection by releasing a range of toxic molecules, enzymes, cytokines and lipid mediators. Finally, natural killer (NK) cells may play a role through release of IFN-γ and through cell-mediated killing. There is considerable redundancy in the effector mechanisms responsible for allograft rejection and attempts to prevent rejection are most likely to be effective if they disrupt the alloimmune response at the level of the T cell rather than at the level of the effector mechanisms themselves.

Patterns of allograft rejection

Three distinct patterns of allograft rejection are recognised. These occur at different times after transplantation and each has a different histopathological appearance.

Hyperacute rejection

This occurs within minutes or hours of transplantation and is due to the presence of preformed antibodies in the recipient. These bind to the vascular endothelium of the graft as soon as it is reperfused with recipient blood. Antibody binding activates complement and also causes type I activation of the vascular endothelium. The net effect is rapid and extensive intravascular thrombosis and irreversible loss of graft function. Hyperacute rejection occurs when an organ is transplanted into a blood group-incompatible recipient and it is essential for all types of organ transplant to ensure ABO compatibility. Hyperacute rejection also occurs when a recipient has preformed cytotoxic alloantibodies directed against HLA class I expressed by the graft. Anti-HLA antibodies may arise following allogeneic blood transfusion, pregnancy or a previous organ transplant. For unknown reasons, kidney allografts are more vulnerable to hyperacute rejection than other types of solid organ transplant whereas liver transplants are relatively resistant. In clinical practice, hyperacute rejection of kidney allografts can be avoided completely by performing a pretransplant cross-match test to make sure that there are no anti-donor HLA alloantibodies present.

Acute rejection

Acute rejection occurs within days or weeks of transplantation and it is not commonly encountered beyond the first six months. It is generally attributed to cellular effector mechanisms although alloantibody may also play an important role. When rejection occurs within the first week it is known as accelerated acute rejection and is a consequence of previous sensitisation to HLA antigens. Acute rejection is usually accompanied by progressive mononuclear cell infiltration of the graft (Figure 43.9). The infiltrate is heterogeneous in nature and includes large numbers of T cells, B cells, activated macrophages and NK cells. Alloantibody deposition may also occur. Initially the cellular infiltrate is predominantly perivascular in distribution but soon it becomes distributed throughout the interstitium of the graft and is associated with a variable degree of graft cell injury. In around 10–20% of cases, infiltration and damage of the graft vasculature is a dominant feature and this is known as acute vascular rejection. Severe acute rejection is characterised by interstitial haemorrhage, necrosis and eventually thrombosis and irreversible graft destruction. The graft becomes swollen and haemorrhagic and may even rupture.

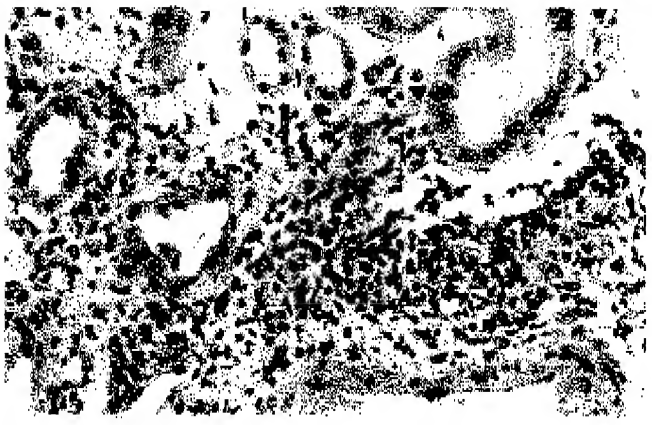


Figure 43.9 Acute renal allograft rejection. There is a heavy interstitial infiltrate of mononuclear cells associated with inflammation of the renal tubules.

In the era before cyclosporin, acute rejection used to be accompanied by pyrexia together with swelling and tenderness of the graft and was a common cause of graft loss. Episodes of acute rejection are still common (occurring in 25–40% of transplants). However, they are not usually associated with such florid clinical signs and in most cases are reversible with supplementary immunosuppressive therapy. Few grafts (<10%) are now lost through acute rejection.

Chronic rejection

Chronic rejection usually occurs beyond the first six months and is the major cause of long-term graft failure. The pathophysiology of chronic rejection is less well understood than that of hyperacute and acute rejection. Alloantigen-dependent effector mechanisms undoubtedly play a key role in chronic rejection but alloantigen-independent factors are also important. The characteristic histological feature of chronic rejection is progressive myointimal proliferation within the arteries of the graft leading to ischaemia, fibrosis and deterioration in graft function (Figure 43.10). Both alloantibodies and cellular effector mechanisms have been shown to trigger chronic rejection and the arterial pathology observed likely represents the stereotypical remodelling response of the arterial tree to injury.

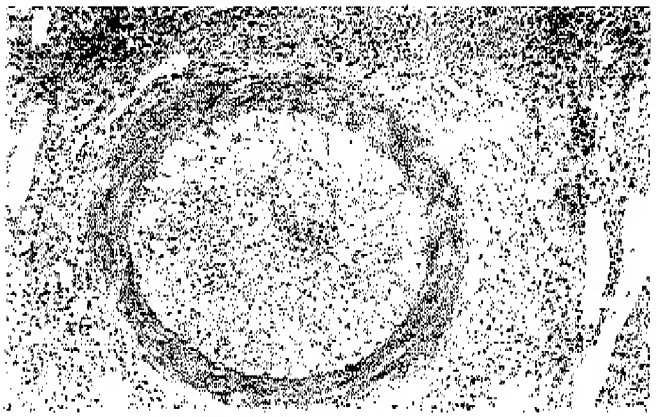


Figure 43.10 Chronic renal allograft rejection. The medium-sized artery shows gross myointimal proliferation causing concentric narrowing and almost complete obliteration of the lumen.

Although chronic rejection occurs months and years after transplantation it is clear that events occurring during the peri-transplant period may have a major influence. In particular, factors that produce early graft injury appear to exacerbate chronic rejection. A number of risk factors for the development of chronic rejection have been identified. Of these, previous episodes of acute rejection that are either multiple or resistant to steroid therapy are the most important. Other risk factors are poor HLA matching, long cold ischaemic time, inadequate levels of immunosuppression and CMV infection. Unlike acute rejection, chronic rejection is largely resistant to treatment with available immunosuppressive therapy.

THE HISTOCOMPATIBILITY LABORATORY

The histocompatibility laboratory has two important roles in clinical transplantation. First, and perhaps most important, the sera of potential transplant recipients are screened for the presence of antibodies which would be detrimental to graft survival. Second, HLA typing of donors and recipients is performed so that matching can be undertaken to reduce the risk of graft loss from rejection. These laboratory investigations are of most relevance to kidney transplantation. In the UK, the histocompatibility laboratory is also responsible for establishing, by means of genetic tests based on DNA variations, the genetic relationship between a proposed living donor and their potential recipient as required by the Human Organ Transplants Regulations 1998.

Antibody screening

Patients awaiting organ transplantation may have developed antibodies to HLA through blood transfusion, pregnancy or a previous organ transplant. In the case of kidney transplantation it is important that a recipient is not given a kidney bearing HLA antigens against which they have developed antibodies. If transplantation were to proceed in the presence of preformed anti-HLA antibodies it would likely result in hyperacute rejection and immediate graft loss. Other types of solid organ transplant are less susceptible to hyperacute rejection but may instead undergo accelerated acute rejection. Liver transplantation can be performed safely in the presence of preformed anti-donor HLA antibodies without hyperacute or accelerated rejection but there may be a detrimental effect on long-term graft survival.

The sera of patients on the waiting list for renal transplantation is routinely screened to determine the presence and specificity of HLA antibodies. Screening of sera should be undertaken before activation on the transplant waiting list and then periodically thereafter. It can be performed in a variety of ways. Commonly, the patients' sera are screened in a microlymphocytotoxicity test using lymphocyte target cells obtained from an HLA-typed panel of donors carefully chosen to represent a wide range of the more commonly encountered HLA antigens. Typically the extent to which a patient is sensitised to HLA is expressed as the percentage of the donor panel against which they have cross-reactive antibodies, so-called 'panel reactivity'. Highly sensitised patients are arbitrarily defined as those who react with more than 85% of the panel cells. A range of commercial assays is now also available for screening sera for the presence of HLA antibodies. These are in widespread use and employ purified HLA class I antigens bound to either microtitre plates or microbeads and rely on ELISA and flow cytometry, respectively, to detect the presence of bound HLA antibody.

When a prospective cadaver or living donor has been identified for a kidney recipient, a lymphocyte cross-match test is routinely performed to ensure the absence of harmful anti-HLA antibodies. In this assay, the most recent recipient serum (along with historical sera) is tested against donor lymphocyte target cells. For many years, all laboratories undertook this analysis using a complement-dependent microlymphocytotoxicity assay in which donor lymphocytes are incubated with recipient sera in the presence of rabbit complement. Antibody binding causes target cell lysis that is detected by microscopy using a dye to distinguish between living and dead target cells. Separated T and B lymphocytes are used as targets in order to detect the presence of class I and class II antibodies respectively. A positive T cell cross-match is a contraindication to renal transplantation. A negative T, positive B cell cross-match is also associated with poor renal allograft outcome if it is due to the presence of anti-HLA IgG antibodies. Often, however, a positive B cell cross-match test arises because of the presence of autoreactive antibodies and these are not detrimental to graft outcome. Since autoantibodies are mostly of the IgM class they can be inactivated by treating the sera with a reducing agent such as dithiothreitol (DTT), thereby allowing a distinction to be made between IgG class anti-HLA antibodies. In sensitised patients, the levels of HLA antibodies may vary over time. So long as a cross-match test using the most recent pretransplant serum sample is negative, renal transplantation can usually be undertaken safely even when a historical serum sample gives a

positive cross-match. Retransplantation is, however, not normally advisable under such circumstances.

Although some laboratories still use the lymphocytotoxic cross-match test, many now use the more sensitive flow cytometric cross-match instead. In this test, recipient antibodies that bind to donor lymphocytes are detected by adding a fluorescent anti-human IgG antibody and then identifying labelled cells by passage through a flow cytometer. Additional staining can readily identify T and B cells, thereby avoiding the need to physically separate them in order to distinguish HLA class I from HLA class II antibodies.

HLA typing

HLA typing used to be undertaken exclusively by serological analysis using the complement-dependent microlymphocytotoxicity test and some centres still use this approach. Carefully selected panels of well-characterised antisera from multiparous women who have developed antibodies against paternal HLA antigens together with monoclonal antibodies directed against defined HLA specificities are used to prepare Terasaki microtitre trays. These are then frozen until required. Serological typing is usually performed on lymphocytes prepared from a sample of peripheral blood. Purified lymphocytes are added to thawed microtitre trays and rabbit complement is added. After incubation an intravital dye is added and the trays are examined by an inverted microscope to determine whether cells in a particular well have undergone lysis, indicating that they have bound antisera against a particular HLA specificity.

The majority of histocompatibility laboratories now perform HLA typing by molecular techniques although a minority still rely on serological methods to type HLA class I. Molecular typing has clear advantages over serological typing. It is usually performed on genomic DNA isolated from peripheral blood cells and because viable cells are not required, patient samples are easier to transport and to store. The oligonucleotide primers used for molecular typing are standard from laboratory to laboratory and can be readily synthesised on demand. Finally, molecular typing provides a much greater resolution of HLA polymorphism than serological analysis and allows identification of HLA specificities that cannot otherwise be detected.

The molecular typing procedures commonly used are all based on the amplification of genomic DNA using specific oligonucleotide primers in the polymerase chain reaction (PCR). There are a number of different approaches. In one approach (PCR-SSOP), DNA encoding the HLA locus of interest is amplified by PCR and the particular HLA allele is then identified by probing the PCR product with a series of labelled sequence-specific oligonucleotide probes (SSOP). Sequence-based typing (SBT) is an alternative approach where PCR is first used to produce a product encompassing the particular HLA allele of interest. The nucleotide sequence of the amplified PCR product is then determined using a DNA sequencer and matched to the known HLA sequences to determine the HLA allotype. A third approach, PCR-SSP, uses a panel of sequence-specific primers (SSP) designed to amplify particular HLA alleles or groups of alleles in the PCR. The PCR products generated are subjected to gel electrophoresis and the resulting pattern reveals the HLA type.

Attempts to reduce the HLA mismatch between organ donor and recipient are made only for kidney and not for other types of solid organ transplant. It is not practicable for heart transplants and in the case of liver transplants appears to offer no immunological advantage.

In the context of kidney transplantation, HLA typing allows detection of HLA antigens in the donor against which a recipient may be sensitised and it allows the opportunity to reduce the mismatch when allocating a cadaveric kidney for transplantation. Mismatches for common HLA antigens should when possible be avoided, especially in young patients, because if the recipient becomes sensitised and graft failure occurs, retransplantation will be made more difficult because of antibodies to a high proportion of potential donors. There is a progressive increase in renal allograft survival as the HLA-A, -B, -DR MM grade decreases from 6 to 0. However, a balance between the practicalities and the benefits of HLA matching is needed. In the USA many centres do not attempt to match cadaveric kidneys for HLA antigens and rely on the use of additional immunosuppression to overcome increased rejection in poorly matched grafts. In the UK an attempt is made to obtain a favourable match, i.e. 0 MM at HLA-DR and no more than 1 MM at HLA-A and/ or -B.

IMMUNOSUPPRESSIVE THERAPY

Historical perspective

The successful development of organ transplantation was to a large extent dictated by developments in immunosuppressive therapy and an understanding of the history of immunosuppression provides a valuable perspective on modern immunosuppressive regimens. Early attempts to prevent rejection of kidney allografts used whole-body irradiation but with few successes and considerable side effects. A breakthrough in the search for chemical agents that could replace irradiation came in 1959 when Schwartz and Damashek found that non-myeloablative doses of 6-mercaptopurine, an anti-cancer drug, were immunosuppressive. Azathioprine, an analogue of 6-mercaptopurine that could be given orally, was then shown by Calne and Murray in Boston to prevent rejection of canine renal allografts. Azathioprine was, when used alone, less effective at preventing renal allograft rejection in humans but it was soon realised that its efficacy improved when it was combined with steroids in the form of prednisone. The combination of azathioprine and steroids gave a one-year renal allograft survival rate of 65–75% and became, for the next 20 years, the standard immunosuppressive regimen for renal transplantation. In 1966, Starzl and colleagues in Denver described the use of polyclonal anti-lymphocyte serum (ALS) as an adjuvant to

azathioprine and steroids. ALS was a valuable addition to existing therapy and provided, when necessary, a means for increasing the level of immunosuppression after renal transplantation. ALS also contributed to the early successes in heart and liver transplantation. In the early 1980s the first monoclonal antibody to be used in transplantation was introduced. This was a mouse antibody (OKT3) directed against the human CD3 molecule found on the surface of T cells and it provided a potent alternative to anti-lymphocyte serum for induction therapy and treatment of steroid-resistant rejection.

The introduction of cyclosporin (cyclosporin A) in the early 1980s was the most significant development since the discovery of azathioprine. Cyclosporin was first discovered during routine screening of fungal extracts at the Sandoz laboratories in Basle and was shown there by Jean Borrel to have immunosuppressive properties. Roy Calne, David White and colleagues in Cambridge, UK, first demonstrated the potential of the new compound as an immunosuppressive agent for organ transplantation and cyclosporin quickly became the mainstay of immunosuppressive regimens around the world. In the UK it was most often used alongside azathioprine and steroids (triple therapy) whereas in North America either ALS or OKT3 was often added as well (quadruple therapy). The use of cyclosporin not only led to a significant improvement in graft survival after kidney transplantation but also facilitated the widespread introduction of heart and liver transplantation.

Towards the end of the 1980s Japanese scientists discovered another fungal metabolite with potent immunosupptessive activity. The new agent, designated FK-506 and later named tacrolimus, was, like cyclosporin, a calcineurin inhibitor and was soon shown by Starzl and colleagues in Pittsburgh to be an acceptable alternative to cyclosporin for kidney and liver transplantation. Some centres, particularly those undertaking liver transplantation, chose to use tacrolimus in preference to cyclosporin-based regimens on account of its potency. The availability of tacrolimus also facilitated early success with small bowel transplantation.

During the late 1990s, the immunosuppressive armamentarium was strengthened further by the addition of several new agents. Mycophenolate mofetil (MMF) was introduced as a more effective alternative to azathioprine after it had been shown to reduce the number of acute rejection episodes when used along with cyclosporin and steroids after kidney transplantation. It quickly replaced azathioprine in most North American centres and many European transplant units. Two new anti-CD25 monoclonal antibodies (a chimaeric and a humanised antibody) were also licensed for use as induction agents for kidney transplantation after they had been shown to reduce the incidence of acute rejection and are in widespread use. Finally, sirolimus, another potent immunosuppressive agent with a mode of action distinct from that of cyclosporin and tacrolimus, was recently licensed for use in kidney transplantation after it too had been shown to reduce the incidence of acute rejection. A number of other new chemical and biological agents are currently undergoing clinical evaluation and when licensed will further increase the choice of immunosuppressive agents for use in organ transplantation.

Immunosuppressive agents

Pharmacological agents

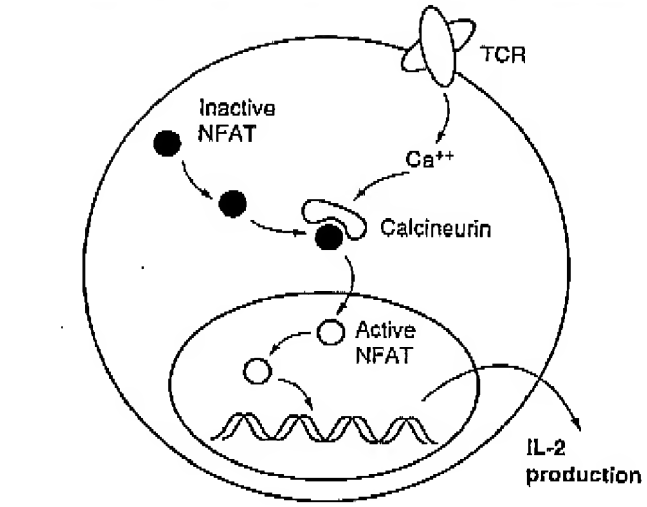
Calcineurin antagonists

A calcineurin antagonist, in the form of either cyclosporin (Cyclosporin A, Neoral®) or tacrolimus (FK-506, Prograf®), is the mainstay of most immunosuppressive regimens for organ transplantation. The two calcineurin antagonists share many similarities but there are also important differences.

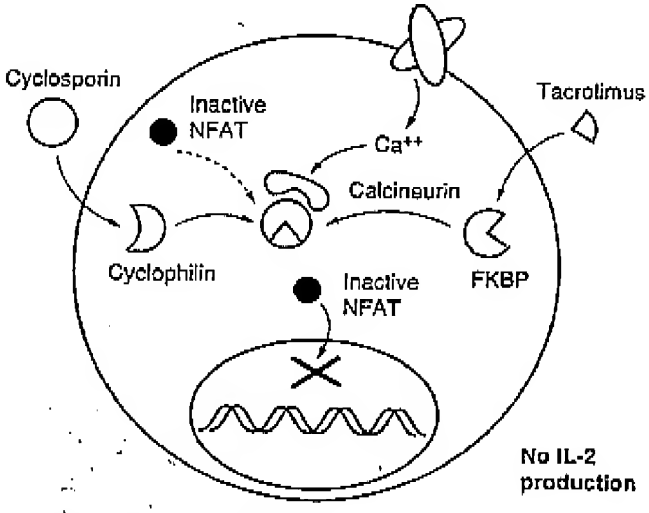
Although cyclosporin and tacrolimus are structurally distinct they have a similar mode of action and their efficacy in preventing allograft rejection is broadly the same. They both exert their principal immunosuppressive effect by inhibiting the activity of the enzyme calcineurin in T lymphocytes, thereby blocking antigen-specific T cell activation (Figure 43.11). After entry into the cytoplasm of the T cell, the drugs bind to their specific receptors or immunophilins. Cyclosporin binds to cyclophilin whereas tacrolimus binds to FK-binding protein (FKBP). The resulting drug/immunophilin complex then binds to and inhibits calcineurin, a calcium/calmodulin-dependent phosphatase. The normal function of calcineurin is to dephosphorylate the cytoplasmic subunit of nuclear factor of activated T lymphocytes (NFAT) after activation of the T cell receptor. This then allows cytoplasmic NFAT to translocate to the nucleus of the cell where it would normally increase transcription of IL-2 and other cytokine genes. By preventing nuclear translocation of NFAT, cyclosporin and tacrolimus block the transcription of key cytokine genes and prevent T cell activation and proliferation.

Cyclosporin was originally produced as an olive oil-based formulation (Sandimmune®) that was dependent on bile salts for absorption and displayed poor and unpredictable bio-availability. A new microemulsion formulation (Neoral®) with improved oral bioavailability, less dependence on bile for absorption and reduced pharmacokinetic variability was introduced in 1995 and has now largely replaced the original formulation. Tacrolimus is well absorbed and absorption is not dependent on bile salts. The mean oral bio-availability is similar to that for cyclosporin at around 30% and, like cyclosporin, there is considerable variability in bio-availability between and within patients.

Cyclosporin and tacrolimus are both metabolised in the liver and gastrointestinal mucosa by the cytochrome P450 (CYP3A4) enzyme system and the metabolites are excreted in the bile. The half-life of the parent compounds is 8–12 hours. Any drugs that induce, inhibit or compete for metabolism by the CYP3A system will have clinically important interactions with cyclosporin and tacrolimus. Cyclosporin and tacrolimus are given in twice-daily doses 12 hours apart. The immunosuppressive activity of the



(A) Normal T cell activation



(B) Effect of calcineurin blockade

Figure 43.11 Calcineurin blockade.

agents is related to their blood levels and it is necessary to monitor these on a regular basis after transplantation in order to ensure optimal therapy and avoid potential side effects. In the case of tacrolimus, the trough level (the drug level immediately prior to the next dose) is a good guide to drug exposure. The trough level of cyclosporin is also frequently used as a guide to drug exposure but some units measure the drug level two hours post-dose (C2 level) instead on the grounds that this is a better indicator of cyclosporin exposure.

Cyclosporin and tacrolimus share several agent-specific side effects but there are also differences in their side-effect profiles. Both agents are nephrotoxic, one of their most serious side effects, and may cause hypertension. Cyclosporin may cause gingival hyperplasia and hirsutism and whilst tacrolimus is largely free from cosmetic side effects, it is more likely to cause post-transplant diabetes and neurotoxicity.

Corticosteroids

Glucocorticoids have a range of anti-inflammatory and immunosuppressive properties and are used extensively as prophylaxis against rejection as well as to treat acute rejection episodes. Most European units prefer to use prednisolone whereas many North American units prefer the 11-keto analogue prednisone. Both preparations have good oral bio-availability. Glucocorticoids enter the cell and bind to intracytoplasmic glucocorticoid receptors. The resulting complexes translocate to the cell nucleus of T cells and macrophages. Here they interact with various glucocorticoid response elements – specific DNA sequences – which modulate the transcription of a wide range of genes. In addition, glucocorticoids mediate indirect effects through inhibition of NFκB. Through these pathways, glucocorticoids act at multiple levels of the immune response. They inhibit the production of several proinflammatory cytokines (including IL-1, IL-2, IL-6 and TNF-α and interferon-γ), impair the function of macrophages and monocytes, interfere with T cell activation and influence lymphocyte recirculation. The side effects of steroids are well known and include hypertension, osteoporosis, diabetes mellitus, dyslipidaemia, peptic ulceration and cushingoid features.

Antiproliferative agents

Most immunosuppressive regimens include either azathioprine (Imuran®) or mycophenolate mofetil (MMF, CellCept®) as a maintenance drug. Until recently azathioprine was the only agent in widespread use. It is an inactive prodrug which after ingestion is rapidly absorbed and converted in the liver to 6-mercaptopurine, a purine analogue. Azathioprine interferes with purine synthesis and inhibits the proliferation of both T and B cells. Its principal side effects arise from marrow suppression, resulting in thrombocytopenia and pancytopenia. The blood count should be monitored carefully and the dose of azathioprine adjusted according to the result. Reversible cholestasis and other gastrointestinal symptoms may also occur with azathioprine. Azathioprine is metabolised by xanthine oxidase. Since allopurinol inhibits this enzyme it markedly increases the bio-availability of azathioprine and increases toxicity.

MMF is the ester prodrug of mycophenolic acid (MPA) and in many centres has replaced azathioprine since it is more effective in reducing acute rejection episodes. The mofetil ester of MPA serves to increase stability and improve bioavailability. After ingestion, MMF is rapidly absorbed and converted to its active form. MPA is a non-competitive and reversible inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH). This enzyme is a rate-limiting step in the *de novo* synthesis of guanosine

nucleotides from inosine. Lymphocytes rely on *de novo* purine synthesis to a greater extent than other cell types because they lack the so-called 'salvage pathway' for purine synthesis. Consequently MPA selectively inhibits the proliferation of both T and B cells. It also inhibits proliferation of arterial smooth muscle cells *in vitro* and this has given rise to hope that it might limit the development of chronic allograft rejection. The side effects of MMF include gastrointestinal symptoms, leucopenia and thrombocytopenia. An enteric-coated form of MMF is in development and may reduce the gastrointestinal side effects sometimes seen with MMF.

Sirolimus

Sirolimus (Rapamycin, Rapamune®) is a macrolide produced by the bacterium *Streptomyces hygroscopis*. It is similar in structure to tacrolimus and binds within lymphocytes to the same cytosolic receptor FKBP-12. Unlike tacrolimus, however, sirolimus is not a calcineurin inhibitor and has little effect on cytokine production. Rapamycin exerts its immunosuppressive effects by inhibiting an enzyme known as mTOR (mammalian target of rapamycin). This intracellular kinase has an important influence on the activity of a number of downstream signalling molecules, including mitogen-activated protein kinase (MAPK). By interfering with signal transduction from the IL-2 receptor and other cytokine receptors, the net effect of tacrolimus is to prevent cytokine-dependent lymphocyte proliferation blocking progression of the cell cycle from the G1 to S phase.

Even though rapamycin and tacrolimus both bind to FKBP-12 the levels are not saturating and hence both sirolimus and tacrolimus can be used together if required. In contrast to the calcineurin inhibitors, sirolimus is not nephrotoxic and does not cause hypertension. Reported side effects include hypercholesterolaemia, hypertriglyceridaemia, thrombocytopenia, impaired wound healing and bone pain. An analogue of sirolimus called SDZ RAD with similar immunosuppressive properties is currently undergoing clinical development.

New pharmacological agents in development

A number of novel small molecule immunosuppressive agents are at various stages of clinical development and some are likely to be licensed for clinical use over the next few years. Promising new agents include FTY720 and the leflunomide analogues. FTY720 is a synthetic analogue of myriocin, a fungal metabolite produced by *Isaria sinclarii*. It is of particular interest because it has a completely different mode of action to existing immunosuppressive agents. FTY720 alters the homing pattern of lymphocytes, diverting them from the peripheral blood into lymph nodes and Peyer's patches. Phase three studies of FTY720 in renal transplantation are currently in progress. Leflunomide, along with other members of the malononitrilamide family, is a potent immunosuppressive agent that affects both T and B cell function. The malononitrilamides block division of activated lymphocytes by inhibiting the enzyme dihydro-orotate dehydrogenase and they also inhibit tyrosine kinase activity. Leflunomide has an excessively long half-life but other family members may prove useful in transplantation.

Biological agents

A number of antibody preparations are available for use as induction agents and to treat rejection episodes. They are only used for limited treatment periods and are unsuitable, therefore, for maintenance immunosuppression.

Polyclonal anti-lymphocyte antibodies

These are produced by immunising animals (horses or rabbits) with human lymphocytes or thymocytes. The gammaglobulin fraction of the serum is purified to yield an antibody preparation directed against a range of lymphocyte cell surface molecules. Polyclonal antibodies are given daily over several days and cause marked depletion of circulating lymphocytes. They commonly cause fever and rigors when first given and occasionally result in anaphylaxis.

OKT3 (Orthoclone®, Muromonab-CD3)

OKT3 is a mouse IgG2a monoclonal antibody directed against the CD3 component of the T cell receptor complex. The antibody binds to T cells, causing rapid depletion of lymphocytes from the peripheral blood. Residual T cells have impaired function because their CD3 complex has either been modulated from the cell surface or is blocked by persistent antibody coating. OKT3 is a potent immunosuppressive agent. When first administered it causes fever, rigors, tachycardia and bronchospasm and in the presence of fluid overload it may cause life-threatening pulmonary oedema. These effects are thought to be due to cytokine release from T cells (cytokine release syndrome) and are lessened by giving an intravenous bolus of methylpredisolone with the first dose. OKT3 may provoke the development of neutralising human anti-mouse antibodies that limit the efficacy of subsequent treatment.

IL-2 receptor antibodies

Two monoclonal antibodies directed against the alpha chain of the high affinity IL-2 receptor (CD25) are now commercially available. They are both mouse anti-human antibodies that have been genetically engineered to make them less immunogenic. Basiliximab (Simulect®) is a 'chimaeric' antibody in which only the variable region of the antibody is of murine origin and the constant region is of human origin. Daclizumab (Zenopax®) is a 'humanised' antibody in which only the antigen-combining site of the immunoglobulin molecule encoded by the six complementarity-determining regions is of murine origin and the remainder is human. Because the alpha chain of the IL-2R is only

expressed after antigen-specific T cell activation, anti-CD25 antibody therapy specifically targets activated lymphocytes (Figure 43.12). Anti-CD25 monoclonal antibodies reduce the risk of acute rejection when given as induction therapy at the time of renal transplantation and they appear to be free from unwanted side effects.

Biological agents in development

A variety of engineered antibodies and fusion proteins directed against key cell surface molecules are under development. These include antibodies directed against molecules such as CD4, CD2 and ICAM-1. There is also intense interest in agents that block the delivery of co-stimulatory activity to lymphocytes through the CD40 / CD40L and the CD28 / B7 pathways and these are entering clinical trial.

Immunosuppressive regimens

Immunosuppressive regimens vary between transplant centres but there are a number of important general principles. The aim in all cases is to provide a level of immunosuppression that is sufficient to prevent allograft rejection. However, excessive immunosuppression should be avoided so as to minimise the risks of infection and malignancy that result from non-specific depression of the immune system. The levels of immunosuppression required after transplantation are highest during the first few weeks when the risk of acute rejection is greatest. Thereafter the level of immunosuppression can be gradually reduced but immunosuppression cannot be stopped completely even after many years without risking graft loss from rejection. It is likely that occasional patients could tolerate very low levels or even no immunosuppression without rejecting their graft but there is no reliable way to identify such patients at the present time.

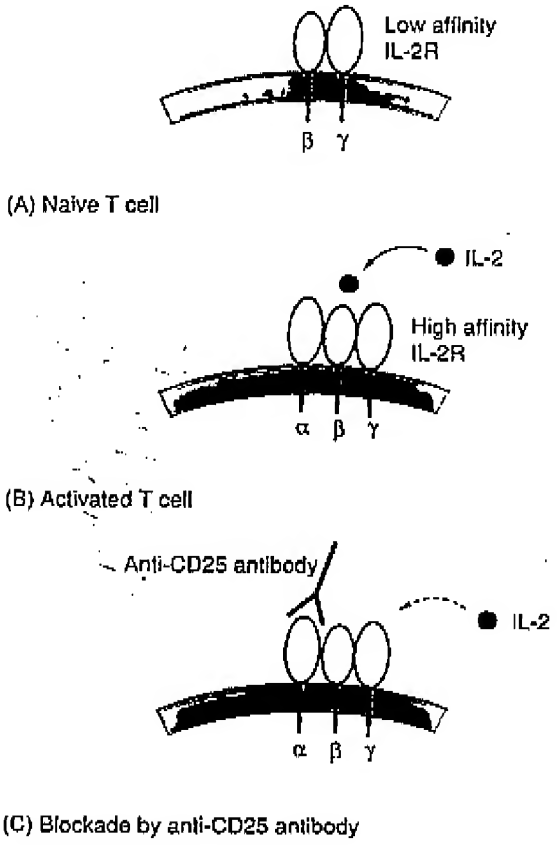


Figure 43.12 Anti-CD25 antibody blockade.

Immunosuppressive regimens are broadly similar for kidney, heart, liver, pancreas and lung transplantation although there is a tendency to give more immunosuppression after thoracic organ transplantation since graft rejection is life threatening. Certain groups of recipients are known to be at greater risk of graft loss from rejection, e.g. recipients who are highly sensitised and are receiving a second or third graft. Such patients may also benefit from higher levels of immunosuppression. Now that there is a widening choice of immunosuppressive agents, the concept of tailoring particular immunosuppressive regimens to individual patients is becoming common.

Prophylaxis of rejection

Most regimens are based on cyclosporin or tacrolimus, given along with an anti-proliferative agent (azathioprine or MMF) and steroids. This combination is known as triple therapy. There is not a clear consensus on whether cyclosporin or tacrolimus is superior. Tacrolimus appears to be a more potent agent than cyclosporin although this is not universally accepted. The choice between the two agents is largely dependent on preference of the unit and the need to avoid agent-specific side effects in a particular category of recipient. For recipients of thoracic organ transplants and for renal allograft recipients judged to be at increased risk of rejection it is common to also include induction therapy using either a polyclonal anti-lymphocyte antibody or anti-CD3 monoclonal antibody (quadruple therapy). The anti-CD25 monoclonal antibodies are also being increasingly used as a standard addition to triple therapy. At the other end of the scale some renal transplant units use dual therapy in the form of a calcineurin antagonist and either an anti-proliferative agent or steroids.

Long-term maintenance immunosuppression is variable. Many centres continue with triple therapy but some reduce and attempt to withdraw steroids completely after several months. The occasional centre attempts to maintain selected recipients on monotherapy with a calcineurin antagonist whereas others attempt to withdraw calcineurin antagonists in patients who have stable long-term graft function, leaving them on an anti-proliferative agent and steroids.

The role of rapamycin in immunosuppressive regimens is not yet clear. It is undoubtedly a potent immunosuppressive agent and can be used to replace a calcineurin antagonist or it can be used effectively alongside cyclosporin and possibly tacrolimus.

Treatment of acute rejection

Acute rejection episodes are treated initially with a course of high-dose corticosteroids given either orally or intravenously over a 3–7 day period. In most cases this will reverse rejection but steroid-resistant and recurrent episodes of acute rejection can be treated with either a polyclonal anti-lymphocyte preparation or anti-CD3 monoclonal antibody.

Infection

All of the immunosuppressive agents used in transplantation cause a non-specific reduction in immunity and leave the recipient at increased risk from infection. Recipients are at particular risk from opportunistic infection, especially viral and fungal infections where protection from infection depends on intact cell-mediated immunity. The overall risk of infection is related to the degree of immunosuppression but is also determined by other factors like the type of transplant, whether the recipient has latent infection and their age, general health and immunological memory of previous exposure to infectious agents. All patients should be carefully screened for infection prior to transplantation and, wherever feasible, infections identified should be eradicated before the transplant procedure is undertaken.

Early recognition together with prompt and aggressive treatment of infection are paramount after transplantation. It is important to remember that the symptoms and signs of infection are often masked or atypical and that infection may be at an advanced stage when diagnosed. It is also important to bear in mind that vaccines may be less effective in patients taking immunosuppressive agents. When possible, pretransplant vaccination is recommended. Non-live vaccines rather than live attenuated vaccines should be given to patients taking immunosuppressive therapy.

Bacterial infections

Transplant recipients are at increased risk from the common types of bacterial infections seen after all forms of major surgery. These include wound infection, intra-abdominal or intra-thoracic abscess, postoperative chest infection and urinary tract infections. The organisms responsible are those that commonly cause infection in other types of surgical patient undergoing major surgery. Bacterial infections usually occur during the first few days and weeks after transplantation. They are more common in those patients who are critically ill before their transplant operation, especially if they are in the intensive care unit with multiple invasive lines and catheters *in situ*. Bacterial infections are also more common in recipients who have a long or complicated postoperative course. It is routine practice to give broad-spectrum antibiotics at the time of organ transplantation to reduce the risk of wound infection and in case there is bacterial contamination of the graft either because of infection in the donor or bacterial contamination during organ procurement and storage prior to transplantation. Acute bacterial infections may be life threatening in the immunosuppressed patient and when identified should be treated aggressively.

Transplant recipients are at increased risk of tuberculosis and chemoprophylaxis should be given to prevent reactivation of disease in those patients who have a past history of tuberculosis or a history of recent exposure. Presentation of tuberculosis may be atypical and for all patients, a high index of suspicion and prompt treatment of suspected tuberculosis are important.

Viral infections

Cytomegalovirus (CMV)

CMV (human herpes virus 5) is one of the most important causes of infection in transplant recipients. Symptomatic CMV disease occurs in up to one-third of recipients of a solid organ and causes considerable morbidity. Before the advent of effective antiviral therapy there was also an appreciable mortality from CMV disease.

CMV is a member of the herpes group of viruses and is widely prevalent in the general population. Around 50% of normal young adults have antibodies to CMV, indicating previous exposure to the virus, and this figure increases progressively with age. The virus is transmitted by direct person-to-person contact through close exposure to body secretions. In a healthy individual with a normal immune system, primary CMV infection is usually subclinical although fever and malaise may sometimes occur. In the presence of immunosuppressive therapy CMV infection can cause a severe and life-threatening disease. CMV disease, as opposed to CMV infection, implies symptomatic or tissue-invasive CMV. Non-specific symptoms including fever, malaise and myalgia are common along with leucopenia, thrombocytopenia and elevated liver enzymes. Depending on the target organ, CMV infection may produce pneumonitis, haemorrhagic gastroenteritis, hepatitis, renal inflammation myocarditis, pancreatitis, retinitis or encephalitis. Infection with CMV may also further reduce cell-mediated immunity and predispose to co-infection with other opportunistic viruses and fungi. The immunological derangement resulting from CMV disease may also be associated with an increased risk of both acute and chronic allograft rejection although this is controversial.

CMV disease after transplantation is most common between one and six months. It may result from a primary infection with the virus in a previously uninfected recipient or it may arise instead from either reactivation of latent virus or reinfection with a different strain of CMV in a previously infected recipient. Recipients who are CMV seronegative at the time of transplantation are most at risk of CMV disease, especially if they receive an

organ from a CMV-seropositive donor, since this is an effective vehicle for transmitting CMV. Primary CMV infection usually presents earlier and is more likely to result in severe disease than CMV reactivation or reinfection. Blood transfusion used to be a common means for transmitting CMV but this is no longer a major problem now that filtered or leucodepleted blood is used routinely.

A range of laboratory methods is available for making a diagnosis of CMV infection and CMV disease. Serology is used to determine previous exposure to CMV in the donor and recipient before transplantation but is of only limited value in the diagnosis of acute infection where the antibody response may be slow to develop. Assays for CMV antigen in cultures of blood and tissue samples are commonly used and the level of CMV antigenaemia is a useful marker of disease progression. Identification and quantification of CMV DNA in blood by PCR are also being used increasingly.

Finally, histological examination of tissue biopsy for viral inclusion bodies and CMV antigen may be useful in the diagnosis of invasive CMV infection.

Adopting a CMV matching policy so that high-risk CMV-seronegative recipients only received organs from CMV-seronegative donors would reduce the incidence of CMV infection in this high-risk group but this approach is seldom adopted because of practical constraints. Vaccination of CMV-seronegative patients prior to transplantation is also an attractive concept but available vaccines have limited efficacy. Passive immunoprophylaxis using hyperimmune globulin provides protection from severe CMV disease and is used in some renal transplant centres. However, most centres use anti-viral agents rather than immunoglobulin as prophylaxis against CMV. These include oral aciclovir, valacyclovir (the L-valine ester of aciclovir) and ganciclovir as well as intravenous ganciclovir. The disadvantage of prophylaxis is that it is expensive, causes side effects and is not always completely effective.

There are two approaches to prophylaxis. The first, known as 'conventional prophylaxis', is to give oral prophylaxis to all recipients or to high-risk recipients. The latter would include CMV-seronegative recipients of a CMV-seropositive organ and recipients treated with anti-lymphocyte antibody preparations. Potential disadvantages are that prophylaxis may merely delay onset of CMV disease until completion of prophylaxis and that it may favour the development of resistant strains of CMV. The second approach, known as 'preemptive prophylaxis', is to monitor recipients regularly, for example by measuring CMV antigenaemia, and to treat them with intravenous ganciclovir when it is predicted they will develop CMV disease. There is, however, no clear agreement on the best way to monitor viral activity and the logistics of CMV surveillance can be considerable.

For treatment of established CMV disease intravenous ganciclovir should be used and consideration should be given to reducing the level of immunosuppressive therapy where this is feasible.

Herpes simplex virus (HSV) and varicella zoster virus (VZV) infection

Reactivation of latent HSV is common during the first few weeks after transplantation. It usually causes ulceration of the lips and mouth but sometimes it produces lesions in the cornea or in the anogenital area. Encephalitis and dissemination to visceral organs are occasionally seen and potentially fatal. HSV pneumonia is sometimes seen in recipients of lung transplants.

Herpes zoster is ten times more common in transplant recipients than in the general population. It is usually seen during the first few months after transplantation and results from reactivation of latent VZV in the sensory ganglion with transport of virus along the sensory nerves to give a vesicular skin eruption in the corresponding dermatome (shingles). Primary VZV infection (chickenpox) is potentially very serious in the immunocompromised patient but fortunately it is uncommon because most adults have immunity through previous exposure to the virus during childhood. If a non-immune patient is exposed to a case of chickenpox, it is advisable to give them VZV immunoglobulin. Prophylaxis and treatment of both HSV and VZV are with anti-viral agents (aciclovir or ganciclovir).

Human herpes virus (HHV)-6, -7 and -8

HHV-6, -7 and -8 are ubiquitous and infection after transplantation may arise from reactivation of latent virus or from transmission of virus in the transplanted organ. The effects of infection range from a brief viral syndrome to tissue-invasive disease. HHV-6 may cause bone marrow suppression and encephalitis and HHV-8 infection has been associated with the development of Kaposi's sarcoma.

Epstein–Barr virus (EBV)

EBV is a ubiquitous herpesvirus. Over 90% of adults have encountered the virus which is either subclinical or causes a mononucleosis syndrome (glandular fever). EBV-seronegative recipients may develop a primary infection following transmission of EBV in an organ from an EBV-seropositive donor and reactivation of latent EBV may occur in EBV-seropositive recipients. EBV targets B lymphocytes, causing them to proliferate and undergo transformation. In an immunocompetent individual the proliferating B cells are held in check by cytotoxic T cells but in the immunosuppressed transplant recipient they may undergo uncontrolled proliferation, leading to post-transplant lymphoproliferative disease.

Human papoviruses (polyoma virus)

Human papilloma virus (HPV) reactivation is common after transplantation and is associated with cutaneous and sometimes anogenital warts. The papovirus family includes BK virus and JC

virus. These are ubiquitous and the majority of healthy adults will have experienced infection during childhood. They remain latent in the kidney and after renal transplantation latent virus in the graft may be reactivated, occasionally causing invasive disease of the graft, bladder and urethra.

Fungal infections

Candida

Oral candidiasis is common after transplantation and candidal oesophagitis is seen occasionally. Topical antifungal agents, e.g. nystatin, are routinely given as prophylaxis. Occasionally candida is responsible for causing deep infections at the site of transplantation and disseminated candidiasis.

Aspergillus

Aspergillus is widespread in the environment and spores are often released in high concentrations during hospital building work. Exposure to aspergillus may lead to the development of aspergillus pneumonia or other forms of tissue-invasive disease. Colonisation is sometimes seen in the absence of invasive disease and chemoprophylaxis is not routinely used in this situation. The diagnosis of invasive aspergillus can be difficult and is often delayed. Treatment is with amphotericin B. Systemic anti-fungal agents are, however, expensive and commonly cause side effects.

Pneumocystis carinii

Pneumocystis carinii is a ubiquitous fungus that causes pneumonia (PCP) in the immunocompromised host. PCP occurs in the first six months after transplantation and presents with fever, dyspnoea and a non-productive cough. Physical and radiological signs may be minimal and the diagnosis is made by bronchoalveolar lavage and transbronchial biopsy. Prophylaxis against PCP after transplantation is routine, usually in the form of trimethoprim-sulphamethoxazole. Without prophylaxis the incidence of PCP is around 10% and much higher after lung transplantation.

Malignancy following transplantation

Organ transplant recipients are much more likely to develop a malignancy than individuals of comparable age in the general population and this is attributed largely to the immunosuppressive therapy they receive. The risk of malignancy after transplantation increases progressively over time and up to half of transplant patients followed for 20 years or more develop a malignancy. The types of cancer encountered after transplantation are markedly different from those seen in the general population. Much of the increase in malignancy is accounted for by cancers of the skin and by lymphomas although there is also a markedly increased risk of urogenital malignancy. When skin cancers are excluded there is a three- to fourfold increase in the incidence of malignancy. Most of the common solid malignancies show a twofold increase in incidence compared to age-matched controls, although there is no increase in carcinoma of the breast or prostate. Malignancy after transplantation occurs at an earlier age than in the general population and the mean age of patients at the time of diagnosis of malignancy is the mid-40s.

The increased susceptibility of immunosuppressed patients to malignant disease is due to multiple mechanisms. Infection with oncogenic viruses undoubtedly plays an important role and the malignancies with the greatest observed-to-expected ratio are those in which viral infection is known or thought to play an aetiological role. Immunosuppression may also enhance the effect of other oncogenic stimuli, for example UV light and carcinogens, and it may impair immunosurveillance and elimination of cells that have undergone malignant transformation. Chronic antigenic stimulation by the allograft may also play a role in the promotion of neoplasia. There is no convincing evidence that any of the commonly used immunosuppressive agents have a significant direct oncogenic effect.

Skin cancer

Cancers of the skin are the most commonly encountered malignancy in transplant recipients and comprise over one-third of all malignancies seen. The majority are squamous cell carcinomas and the normal preponderance of basal cell carcinoma over squamous cell carcinoma in the general population is reversed after transplantation. Exposure to sunlight is a major aetiological factor and squamous cell carcinoma is particularly common in white Caucasian recipients exposed to strong sunlight. Many of the malignancies occur on sun-exposed areas of the body although non-exposed areas including the vulva and vagina are often affected. Squamous cell carcinomas after transplantation are often preceded by premalignant lesions and are commonly multiple. They tend to grow rapidly and are more likely to metastasise and recur than lesions occurring in the general population. Reactivation of HPV has been implicated as an aetiological factor in the development of squamous cell carcinoma.

Transplant recipients should be advised to protect their skin from exposure to strong sunlight and should be assessed regularly for the presence of premalignant and malignant skin lesions. When identified, these should be treated promptly and aggressively. Consideration should be given to reducing the level of immunosuppressive therapy in patients with multiple or rapidly developing skin cancer.

Post-transplant lymphoproliferative disease (PTLD)

After skin cancer, PTLD is the next most common malignancy seen in transplant recipients and affects between 1% and 5% of recipients. The majority (95%) of cases of PTLD are associated with EBV infection and the incidence is highest in recipients who

have received a high total burden of immunosuppression. EBV-seronegative recipients of an EBV-seropositive organ allograft are at particularly high risk of developing PTLD and the incidence of PTLD is greatest in paediatric recipients. The disease progresses with varying rapidity through a clinical spectrum ranging from EBV-driven polyclonal B cell activation and hyperplasia to malignant monoclonal B cell lymphoma. PTLD may be confined to secondary lymphoid tissue or it may develop at extranodal sites including the gastrointestinal tract, central nervous system and organ allograft.

The presentation may be varied. Pressure symptoms from an enlarging mass are a common presentation. A high index of suspicion aids early diagnosis. Reducing or, where feasible, even stopping immunosuppression is the most important treatment and may lead to the complete regression of the disease. Localised disease may be amenable to surgical excision or radiotherapy. Anti-viral drugs and IFN-α may also be used. If the disease does not respond promptly to treatment the outlook is usually poor.

Genitourinary cancer

Cancer of the genitourinary tract is common after transplantation, particularly in the female. There is a markedly increased incidence of carcinoma of the uterine cervix and this is associated with HPV infection. Regular screening and early treatment are important.

Kaposi's sarcoma

Kaposi's sarcoma is rarely seen in Western countries except in patients with HIV infection. After organ transplantation there is a 100-fold increase in the incidence of Kaposi's sarcoma, although it still remains uncommon, affecting <0.5% of recipients. It is associated with HHV-8 infection and recipients of Mediterranean origin are particularly susceptible to developing the condition. It usually presents as a cutaneous lesion but may also involve the visceral mucosa. If immunosuppression is reduced or stopped the disease may completely regress but disseminated disease is usually fatal. Anti-viral agents may be beneficial.

FUTURE PROSPECTS IN TRANSPLANTATION

Chronic rejection is the major cause of graft failure and there is a need to better understand the pathophysiology of this condition and to develop effective approaches for preventing it. The shortage of human organs for transplantation means that it is essential to optimise graft survival. For many years a major goal in transplantation has been to develop strategies whereby the recipient is made specifically tolerant to their transplant without the need for non-specific immunosuppressive therapy. A state of transplant tolerance would ensure long-term graft survival and leave the recipient free from all the complications of immunosuppressive drugs. It has long been known that specific immunological tolerance to organ allografts can be achieved by various methods in experimental animals but achieving transplant tolerance in humans has proved very elusive. One of the more promising approaches for inducing tolerance is the use of monoclonal antibodies directed against molecules that provide essential co-stimulatory signals for T cell activation. Early clinical trials of such antibodies, notably those interfering with CD40–CD40 ligand interaction, are currently in progress but it is too early to assess the efficacy of this approach.

The use of animal organs for transplantation into humans is a potential solution to the shortage of human organs but very considerable problems have to be overcome to make this approach safe and feasible. The use of non-human primates as a source of donor organs is not acceptable by most people because of ethical concerns. Moreover, primates are difficult to breed in captivity and may harbour pathogens that are dangerous for humans. Consequently the pig has emerged as the most suitable potential source of donor organs on the basis of size, physiology and ease of breeding. There are still ethical concerns, however, as well as concern about the potential for transmission of pathogens such as porcine endogenous retrovirus. This virus is embedded within the pig genome and cannot, therefore, be eliminated as a potential hazard.

Until recently hyperacute xenograft rejection was a major barrier to the successful transplantation of pig organs in primates. Most primates, including humans, have naturally occurring IgM and IgG antibodies directed against a carbohydrate antigen, galα(1,3)gal, expressed by pig tissues. This is analogous to a blood group antigen and causes hyperacute rejection and xenograft destruction within minutes of performing xenotransplantation. However, hyperacute xenograft rejection of pig organs in primates can now be overcome using a variety of approaches. Of these, one of the most effective is to use organs from pigs that have been made transgenic for human complement regulatory proteins such as decay accelerating factor. Considerable immunological barriers still remain, however. Accelerated vascular rejection occurs within the first three or four days and if this is overcome acute rejection and probably chronic rejection are major barriers. Conventional immunosuppressive agents are not sufficiently potent to prevent these processes without causing unacceptably high risks from infection because of over-immunosuppression.

FURTHER READING

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